THE CHEMISTRY OF COMPONENTS ISOLATED FROM KERATIN FIBRES

by

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I certify that all the work presented in this thesis is original, except where due credit is given to the work of others.

L.C. Chapman

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SYNOPSIS

Following an outline of the structure and composition of keratin fibres, the literature related to the ortho- and para-cortices in keratin fibres, is reviewed.

The development of a method for removal of cuticle from keratin fibres, using a Vibromix agitator, is described. Amino acid analyses of five keratin fibres [Merino wool, two different Alpaca fibres (one pigmented and the other unpigmented), Human hair and Possum fur] and their cuticles were performed. Conclusions drawn from these analyses are (i) the differences between the cuticle and fibre compositions suggest that the cuticles are amorphous due to their amino acid composition, (ii) the cuticles are less polar than the fibres and (iii) citrulline is present in the cuticles as a constituent amino acid and not absorbed into the protein.

After staining some Merino wool fibres bilaterally with gold, the fibres were dispersed using ultrasonics and the ortho- and para-cortical cells separated on density gradients. Amino acid analyses and high- and low-sulphur analyses were performed on the separated cortical cells. The compositions of the ortho- and para-cortical cells were found to be almost identical. A possible explanation of the different dyeing properties and chemical stability of the two cortices is put forward.

CONTENTS

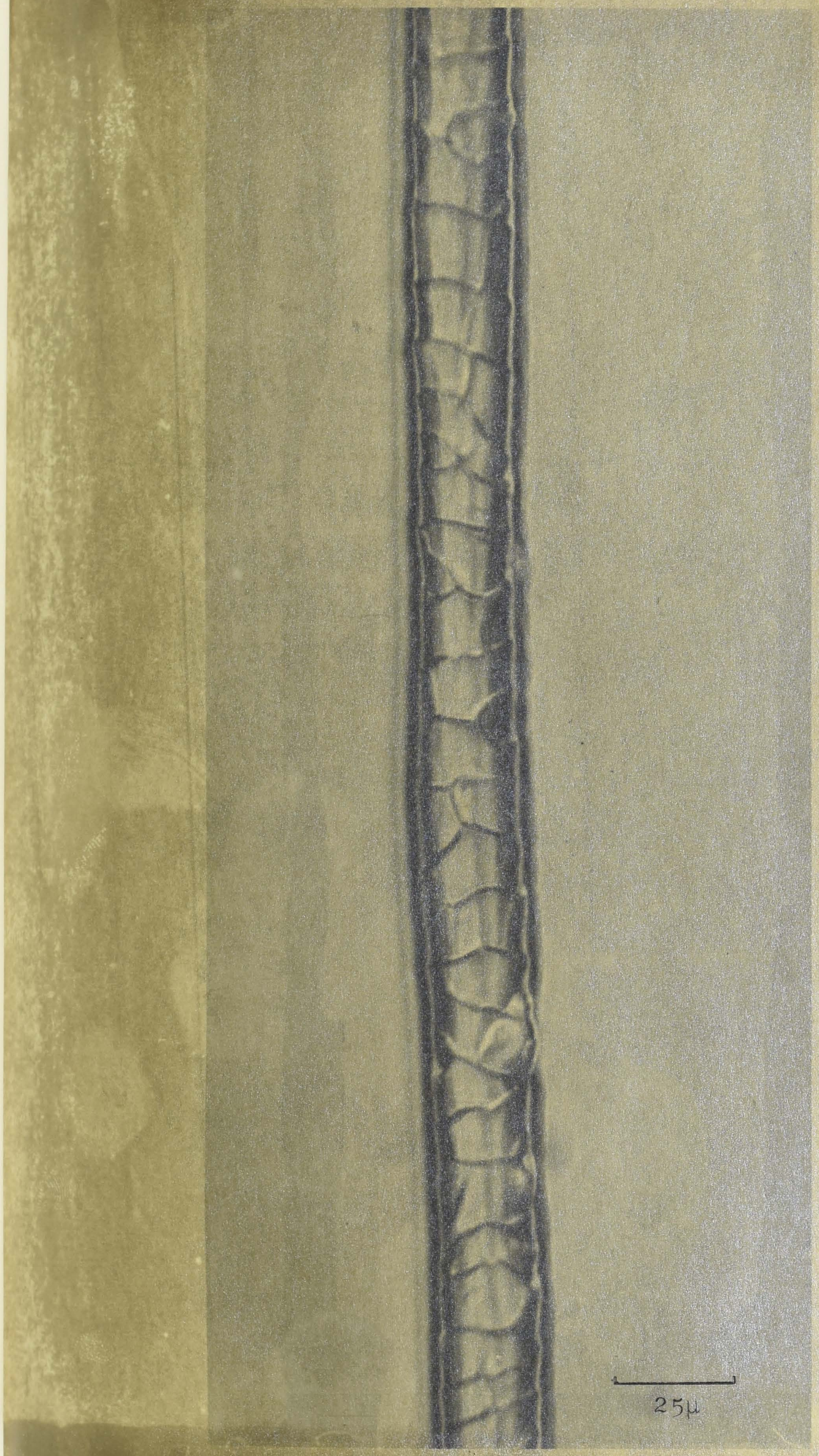


Figure 1.1.
Replica of a wool fibre.

1. SUMMARY OF THE KNOWN HISTOLOGICAL STRUCTURE OF KERATIN FIBRES

[A] MAJOR FEATURES OF THE STRUCTURE.

The chemical and physical structure of keratin fibres, has been summarized in four excellent reviews published recently [Lundgren and Ward (1962, 1963), Ryder (1963) and Crewther et al. (1965)]. However a summary of the histological and chemical structure of keratin fibres is pertinent.

Until recently, there has been some confusion in the literature concerning nomenclature of the structural components of keratin fibres. Mercer et al. (1963) proposed a nomenclature which, if used by all workers carrying out research on keratins, will avoid confusion. Johnson and Sikorski (1965a) disagree, stating that the new term 'filament', which replaces the old term 'microfibril', is unfortunate.

They point out that the meaning of the term 'filament' has long been established as "a fibre of indefinite length" [Textile Terms and Definitions, Textile Institute (1954)]. However, there is no likelihood that the meaning of the term would be confused or cause any concern when referring to the fine structural component of wool and consider the term 'filament' to be more suitable than 'microfibril'. Therefore the nomenclature proposed by Mercer et al. (1963) will be used throughout this thesis.

It must be stressed that when discussing the chemical, physical and histological structure of keratin fibres, known details come mainly from investigations of the structure of the wool fibre. Most keratin research has been carried out on wool due to its abundance and more particularly to its economic value. However, there is considerable evidence that most of the findings will be applicable to the other keratin fibres with only minor variations [Ryder (1963)].

The keratin fibre consists of two main components, the cuticle and cortex [see figure 1.2]. In wool there is 10% cuticle, 88% cortical cells, with the other two percent being cell membranes [Bradbury and King (1967)]. The cuticle is made up of individual flat cells which overlap each other and form a protective covering around the cortex or central part of the fibre, as can be seen in figure 1.1. The cortex, consists of cortical cells in the case of fine fibres, but in coarser fibres, a central core of medullary cells may also be present.

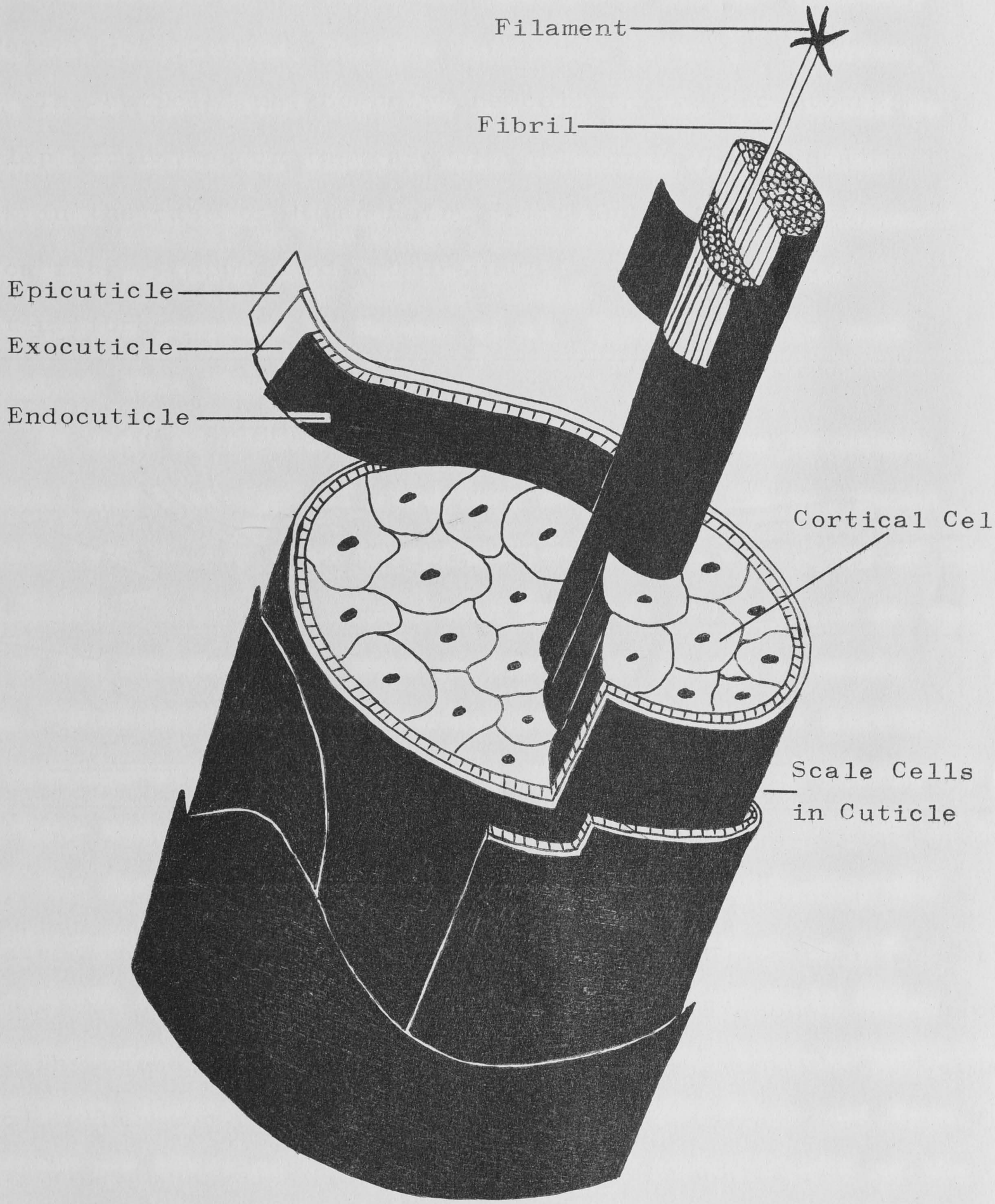


Figure 1.2. Diagram of the histological structure of a keratin [wool] fibre. [From Rogers (1959a)].

Medullary cells are not keratin, but consist of a protein with its own characteristic composition which is termed tricohyalin [Rogers (1964)].

The cuticle, may be only one cell thick, except where overlap occurs, as in the case of wool, or maybe several cells thick in the case of Human hair [Swift and Holmes (1965)], Alpaca fibres [Appleyard and Greville (1950)] and possibly other keratin fibres.

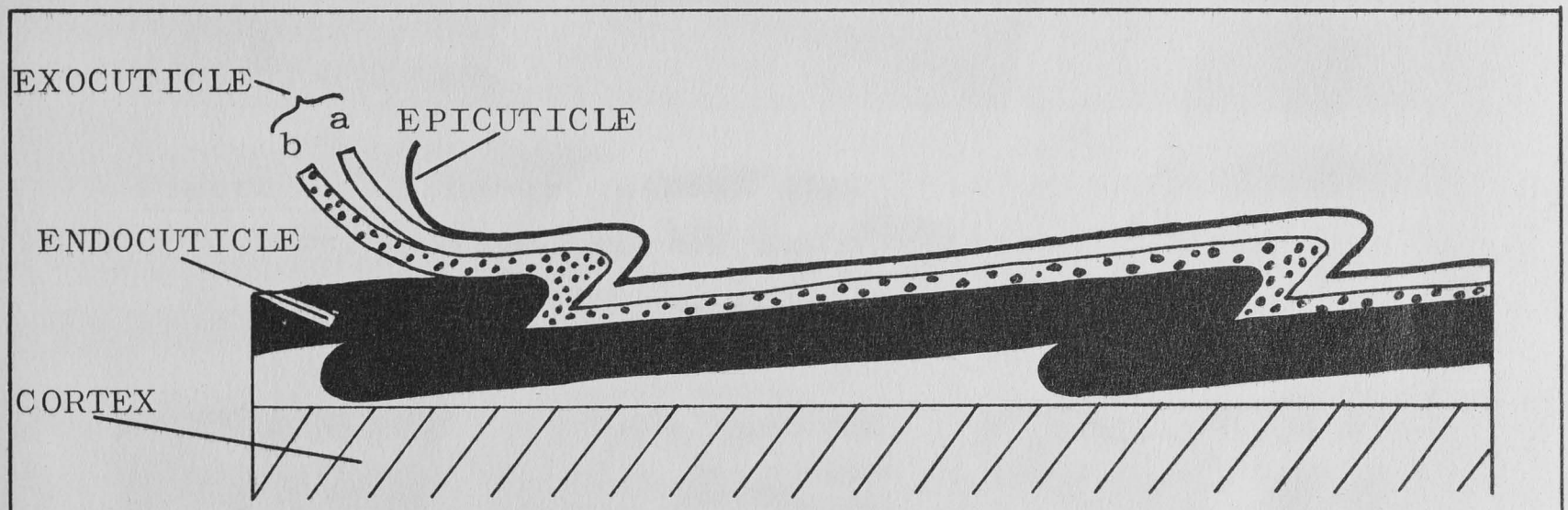


Figure 1.3 Diagram of the Structure of Cuticle. [From Fraser and Rogers (1955d)].

The structure of the cuticle is shown in figure 1.3 and figure 1.4 is a phase-contrast light micrograph of cuticle isolated from wool by the method of Bradbury and Chapman (1964), showing that it consists of flat plates, roughly square in shape.

The presence of the epicuticle was first shown by Allworden (1916). When wool is treated with freshly prepared chlorine water or bromine water, "Allworden sacs" or bubbles appear on the surface of the fibre. The epicuticle acts as a semipermeable membrane. The chlorine water [or bromine water] penetrates through the epicuticle and reacts with the underlying layers

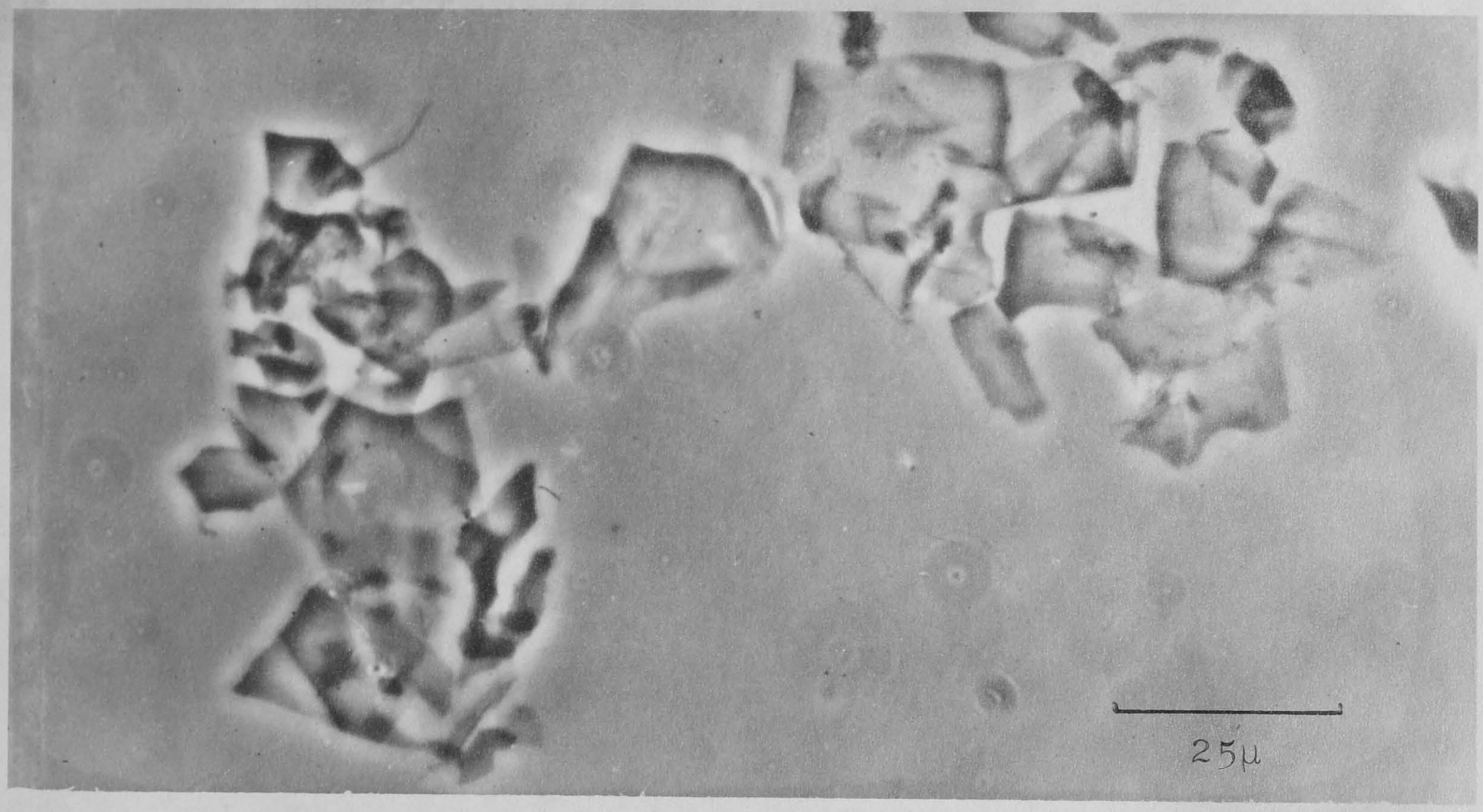


Figure 1.4. Phase-contrast light micrograph of cuticle isolated from wool by ultrasonic disintegration in formic acid.

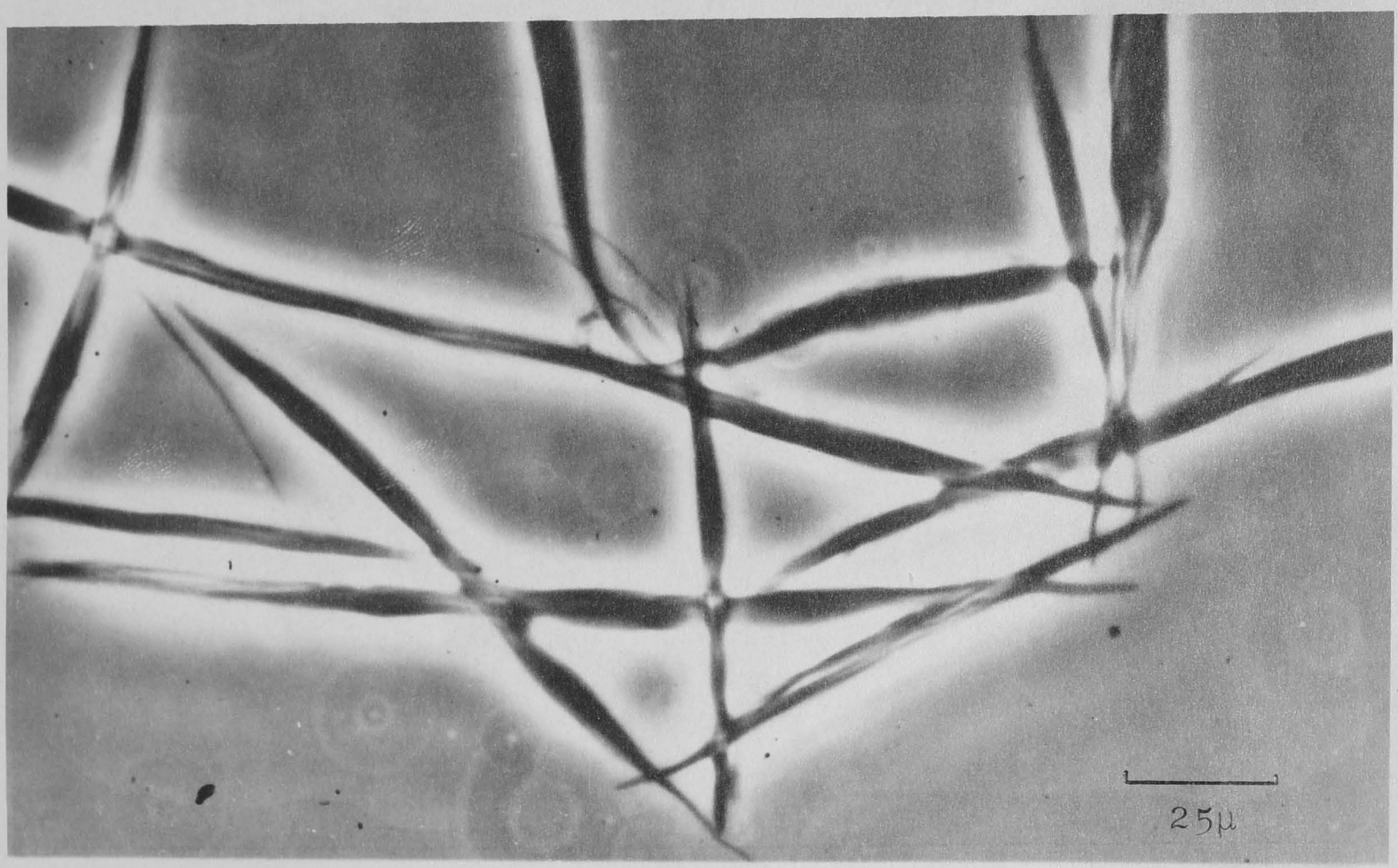


Figure 1.5. Phase-contrast light micrograph of cortical cells produced as in figure 1.4.

of the cuticle, dissolving some of the protein, which is unable to pass out through the membrane. The resulting osmotic pressure forces the membrane out and forms the bubble [Fraser and Rogers (1955d)].

The exocuticle is non-fibrous and readily attacked by keratinolytic reagents, whereas the endocuticle, the innermost layer of the cuticle, is susceptible to enzyme attack [Lagermalm (1954), Fraser and Rogers (1955 b)]. When cross-sections of the cuticle, stained with OsO_4 after reduction with thioglycollic acid [Rogers (1959b)], are viewed in the electron microscope, a third component is revealed. This is the "a" layer of the exocuticle [see figure 1.3] which stains more heavily than the other components of the cuticle.

Up to 1965, no complete analysis of cuticle had been reported, and the partial analyses that were, [Geiger (1944a, 1944b), Lustig, Kondritzer and Moore (1945), Lindley (1947), Ward, Binkley and Snell (1955), Derminot and Leveau (1956), Elliott and Roberts (1957), Derminot (1958), Leveau (1958), Elliott, Asquith and Rawson (1959), Bradbury (1960)], show large differences between the results of the different workers. The differences are due to unknown amounts of chemical degradation by the method of isolation, and in some cases, possible lack of identification or purity of the sample analysed. Using ultrasonics [Bradbury and Chapman (1964)], Bradbury, Chapman and King (1965a) isolated and performed amino acid analysis on cuticle from wool fibres. This was the first complete analysis

of the cuticle. They also showed that there was no possibility of chemical degradation of the cuticle by their method of isolation. Subsequently, a method for removal of cuticle using a Vibromix agitator was developed and is reported herein. Using this method, the amino acid analyses of cuticle from Human hair, Possum fur, two samples of Alpaca [reported herein] and from Bibrik [a Pakistani carpet wool], Mohair and Rabbit fur [King (1967)] have been performed. Andrews, Inglis and Williams (1966) have also analysed cuticle removed from shrinkproofed wool.

Bradbury et al. (1966) have shown, for the first time, the existence of the amino acid citrulline in the cuticle of wool and the cuticle of the keratin fibres outlined above [see also section 2 C]. Rogers (1964) showed that citrulline is present in the inner root sheath and medulla of keratin fibres, and these are the only confirmed reports of this amino acid occurring in natural proteins.

The cortex of fine keratin fibres consists of cortical cells. These are about 80 - 100 microns long and 5 - 10 microns in diameter. Cortical cells isolated from wool by the method of Bradbury and Chapman (1964) are shown in figure 1.5.

The cortex of fine wool fibres can be bilaterally divided into two segments, the ortho- and para-cortex, differing in chemical stability and dyeing behaviour [see section 1B]. Other keratin fibres also show cortical differentiation, but not necessarily bilateral. The coarser fibres, in general, show

radial asymmetry. In particular, the asymmetry of fibres which have been investigated is shown in table 1.1.

Electron microscopic studies of hair, wool and quills have revealed further structural details of the cortex. The revelation of the fine structure of keratin fibres was hindered, until Rogers (1959b) developed a suitable staining procedure. This consisted of partially reducing the fibre with thioglycollic acid and then staining with osmic acid [referred to as the TGA - OsO_4 procedure].

The cortical cells are separated by cell membranes approximately 250 Å wide. The membrane has a central dense layer [about 150 Å] and a less dense region on either side of it [Rogers (1959b)].

Within the cortical cells, fibrils can be seen which consist of masses of filaments embedded in a "cementing" matrix. At this level of magnification, the ortho- and para-cortex can easily be seen [e.g. see Rogers (1959 a,b)].

The fibrils in the orthocortex are smaller in diameter than in the paracortex. This is probably due to more cytoplasmic debris, which separates the fibrils, being found in the orthocortex, but is also due to the closer packing of filaments in the orthocortex compared with the paracortex.

The filaments, which are 80 Å in diameter [Filshie and Rogers (1961)], are separated from each other by the intrafibrillar matrix which is considered to be an amorphous sulphur-rich protein [Rogers (1959b)].

TABLE 1.1.

ASYMMETRY OF VARIOUS KERATIN FIBRES[a]

FIBRE	TYPE OF ASYMMETRY		
	Bilateral	Radial[b]	Other
Wool -			
Merino	X		
Rambouillet	X		
Hardwick	X		
Romney	X		
Welsh Mountain	X		
Portugese Seragosa	X		
Yorkshire grown Leicester	X		
Leicester-Dalesbred	X		
Copper-deficient "steely"	X		
Kerry Hill	X		
Mouflon	X		
Corriedale	X	X [para]	
Doggy [or "anomolous"]	X	X [para]	X [+6 others]
Pakistani carpet	X	X [para]	X [+ 7 others]
Crimpless mutant		X [para]	
Buenos Aires fleece		X [para]	
Lincoln		X [para+ortho]	
Burmese		X [para]	X [no differentiation]
Human hair [straight]			X [all para]
Negro hair [crimpy]	X		
Vicuna	X		
Cashmere	X		
Red deer underhair	X		
Dog underhair	X		
Sloth hair	X		
Albino mouse and rat hair	X		
Alpaca [Huacayo]	X		
Alpaca [Suri]		X [para]	
Mohair		X [para]	
Lion whiskers		X [ortho]	

[a] See section 1B (iii) for further details and references.

[b] Cortical segment indicated refers to the peripheral segment.

The fine structure of the filaments was first revealed by Filshie and Rogers (1961), who post-stained sections of TGA - OsO_4 stained wool with $Pb(OH)_2$ and later with $KMnO_4$ [Rogers and Filshie (1962)]. They found that within each filament there were protofilaments, again embedded in an electron dense matrix - the intrafilamentous matrix- which is also thought to be sulphur-rich. They suggested that there were nine protofilaments peripherally arranged around a stained central region containing two more protofilaments. The "9+2" arrangement was thought to be the most likely, since it bore a strong resemblance to the structural organization in animal cilia and bacterial flagella.

The "9+2" structure has been disputed by Johnson and Sikorski (1962, 1965a), who state that as yet, it is not possible to assign any unequivocal structure to the filament from electron microscopic evidence alone. Also, the diameter of the filament, 80 Å, is too small to accommodate the "9+2" protofilament structure, and the density calculated from this structure would be too low for the native keratin. They have concluded that a unique model for the filament, based on electron microscopic evidence alone, appears unlikely [Johnson and Sikorski (1965b)].

The exact substructure of the protofilament is in doubt, but consists of two or three α-helices in the form of a coiled-coil. Both of these models are consistant with X-ray data [Crewther et al.(1965)], but the triple α-helical coiled-coil

model appears to be the most favoured.

Recently, Dobb (1964, 1965), claims to have observed what could be α-helices from wool. He found that iodinated wool, treated with ultrasonics, was dispersed into filaments and protofilaments. Some of the protofilaments frayed further into three segments each 10 Å wide. He considered these to be α-helices. Johnson and Speakman (1965) have also made similar observations, but it is too early to consider that the evidence is conclusive.

Again, up to 1965, only incomplete analyses or analyses which are suspect, have been reported for cortical cells [Mercer, Golden and Jeffries (1954), Golden, Whitwell and Mercer (1955), Ward, Binkley and Snell (1955), Derminot and Leveau, (1956), Leveau (1957, 1958, 1959e), Derminot (1958), Simmonds and Bartulovich (1958), Schoberl (1960), Leach, Rogers and Filshie (1964), Haly and Inglis (1964), Parisot, Allard and Baures (1965), Miro and Blade (1965), and Horio et al.(1965)]. All these analyses concern either orthocortical cells, paracortical cells, or both, and were performed in an effort to elucidate the differences, if any, between the two cortices. For more details of the analyses see section 3D(ii).

Bradbury, Chapman and King (1965a) have reported amino acid analyses of chemically unmodified cortical cells mechanically disrupted from wool. Their analyses were [within experimental error] the same as for the whole fibre, but this is not surprising since the cortex comprises approximately 88% of the fibre [Bradbury and King (1967)].

Attempts to isolate a pure protein from wool, for confirmational and sequence studies, is proving a difficult task. The nature of keratin, with its interchain disulphide linkages, means that some form of chemical degradation has to be performed on the native protein to break the disulphide bonds, before the keratin can be solubilized and fractionated.

Promising results have been obtained by two methods. (a) Reduction followed by alkylation of the free -SH groups with iodoacetate and (b) oxidation with performic acid or peracetic acid, followed by extraction with dilute ammonia.

The reduction method has been developed by Gillespie and co-workers [see review by Crewther et al.(1965)] using potassium thioglycollate in 6-8M urea solutions at pH 10-11. The resulting solution of protein after alkylation can be separated into two main components called the "kerateines". One component having a sulphur content lower than wool, called the low-sulphur protein and designated SCMKA [S-carboxymethyl-kerateine A], is precipitated at pH 4.4 [Gillespie, O'Donnell and Thompson (1962)], while the other component having a sulphur content higher than wool, the high-sulphur protein, designated SCMKB [S-carboxymethyl-kerateine B], remains in solution.

Fractionation of both the high-and low-sulphur proteins into two fractions has been accomplished , $SCMKA_1$ [Gillespie (1958, 1960)], $SCMKA_2$ [Gillespie and Lennox (1953, 1955)], $SCMKB_1$ [Gillespie (1962)] and $SCMKB_2$ [Gillespie (1963a)]. These fractions still do not represent homogeneous proteins.

The $SCMKA_2$ fraction can be separated into at least five major slow bands and traces of faster moving bands, using starch-gel electrophoresis [Thompson and O'Donnell (1964)]. The SCMKB fraction has also been shown to contain at least eight proteins, using moving boundary electrophoresis and chromatography on DEAE-cellulose [Gillespie (1959, 1963b)].

The treatment of wool with peracetic acid [Alexander and Earland (1950)], or preferably performic acid [Thompson and O'Donnell (1959)], oxidizes the sulphur in the disulphide bonds to sulphonic acids. The resulting "keratose" can then be fractionated into three components, after extraction with ammonia solution; α-keratose is precipitated by acidification of the solution to pH 4.4, ϒ-keratose remains in solution and the insoluble residue is termed β-keratose. The α-keratose has a lower sulphur content than wool and resembles the SCMKA proteins, and the ϒ-keratose has a higher sulphur content than wool and is similar to the SCMKB proteins.

The origin of the extracted proteins mentioned above [SCMKA, SCMKB, α-, β- and ϒ-keratoses], has not been definitely established. There is evidence to show that the low-sulphur proteins are derived from the crystalline part of the fibre - the filaments - and the high-sulphur proteins are derived from the amorphous matrix proteins [see Crewther et al. (1965) and Rogers (1964)]. The β-keratose, and material remaining undissolved after extraction of the SCMK protein fractions, is composed of cell membrane and nuclear remnant material.

Asquith and Parkinson (1966) have found that cuticle from Human hair is composed of only ϒ-keratose. This is consistent with results of other investigations of the cuticle using X-rays [Woods (1938), Lustig, Kondritzer and Moore (1945)] and amino acid analyses [Bradbury, Chapman and King (1965a)] which show that the cuticle is amorphous. Isolated cortical cells from Human hair showed some content of α-keratose and slightly higher content of ϒ-keratose compared with the whole fibre, whereas cortical cells from wool had a higher α-keratose content and lower ϒ-keratose content than the parent fibre.

Gillespie and Inglis (1965) have reported the high-sulphur protein content of a variety of α-keratins and have found that the content varies from 45% in Racoon hair to 7% in Rhinoceros horn. The amount of low-sulphur proteins in these keratins has not been reported except on the basis of subtracting from 100 gm the sum of the weight of high-sulphur protein and an assumed weight of 10 gm of membranes and nuclear remnants [Gillespie (1965)]. Thus they range from 45% in Racoon hair to 73% in Rhinoceros horn.

In the larger diameter fibres, the cortex no longer consists only of cortical cells but also has a central core of medullary cells.

A description of the formation of the medulla in the follicle has been given by Auber (1952). It forms from the cells around and above the papilla of the bulb in the follicle [see figure 1.7]. The amorphous protein of the medullary cells,

during desiccation,agglomerates into large masses and intercellular gaps appear due to insufficient protein material to adequately fill the cells.

The medulla is resistant to alkali and keratinolytic reagents [Mercer (1961)], as evidenced by the method of isolation, using alkali to dissolve all of the fibre except the medullary cells [Matoltsy (1953), Ross and Speakman (1957)]. Mercer (1961) considers that these methods of isolation would undoubtedly alter the protein.

Rogers (1962) has analysed medullary cells isolated from Rabbit fur and from the quills of African and North and South American porcupines, and found that it does not resemble "hard keratins" e.g. fibres. He has shown the existence of the amino acid citrulline, in the protein, in relatively large quantities [Rogers (1962)]. In agreement with others [Blackburn (1948), Matoltsy (1953), Rudall (1955b)],the cystine content is very low, in fact undetectable in his samples.

Both Rogers (1964) and Mercer (1961) have stated that the medullary protein resembles trichohyalin, the protein of the inner root sheath [a peculiar protein which initially forms as amorphous droplets, but later is converted into a fibrous form]. Mercer (1961) considers that the presence of citrulline in a protein could be diagnostic of the protein trichohyalin.

Keratin fibres which grow from the skin are produced in follicles as shown in figure 1.6. The root of the fibre is alive and soft, but when it becomes hardened during keratinization, it dies.

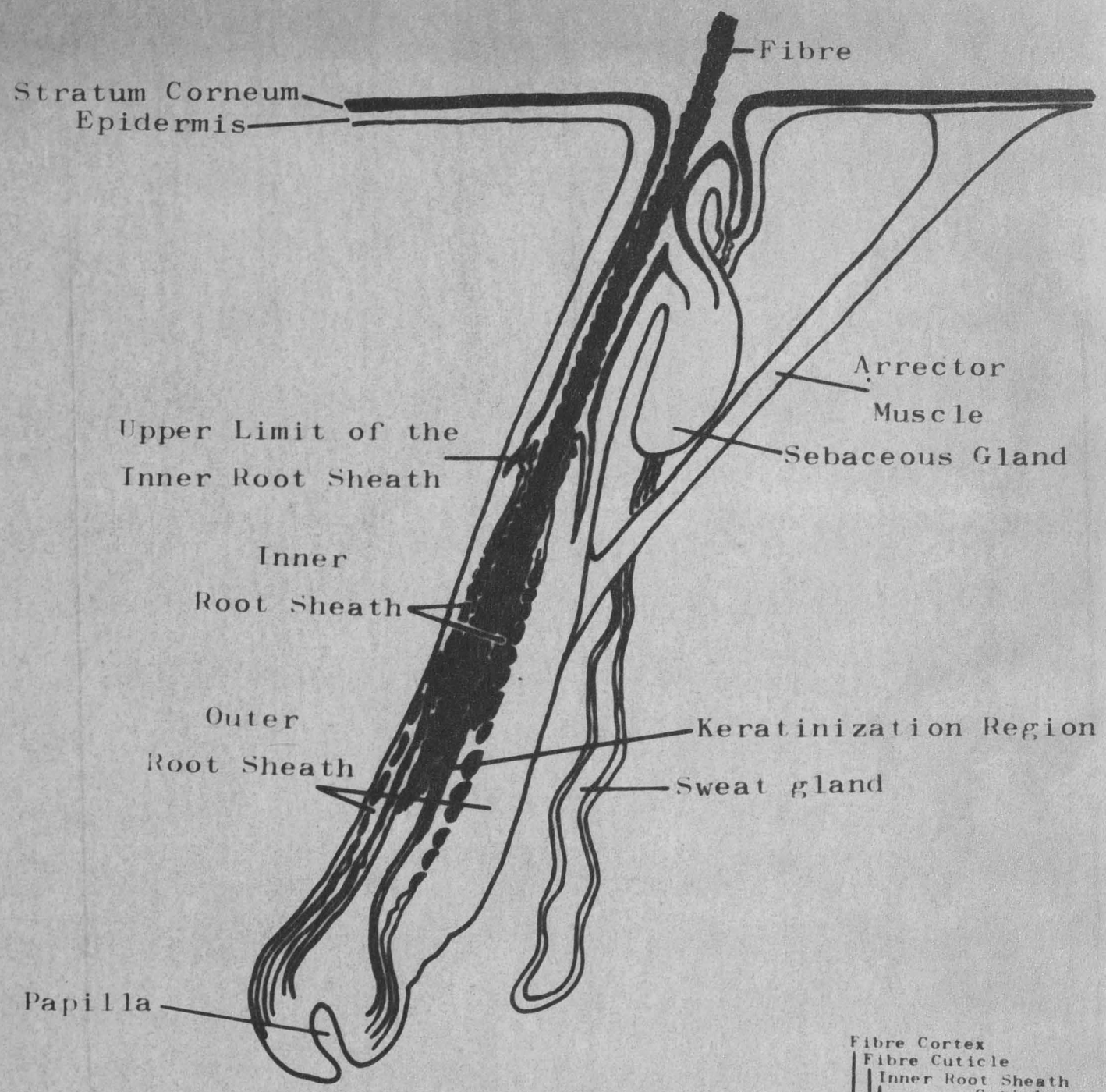


Figure 1.6.

Diagram of a primary wool follicle
[From Auber (1952)].

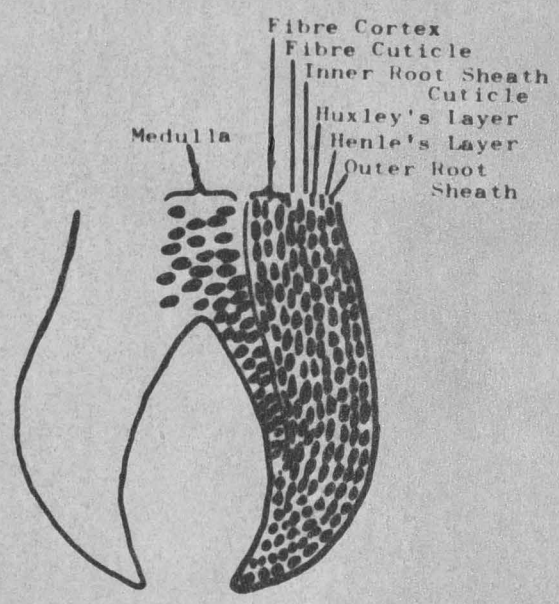


Figure 1.7.

Diagram of the bulb of a wool follicle
showing differentiation.
[From Auber (1952)].

Cells are produced in the bulb of the follicle, and are polyhedral in shape with rounded nuclei. The cells, which are actively dividing in this region, are slowly pushed up the follicle. Towards the top of the bulb they become differentiated and form the various layers in the follicle [see figure 1.7]. As the cells are pushed further up the follicle, the cortical cells become elongated and the cells which will form the fibre cuticle become flattened.

At about a third of the way up the follicle, the cortical cells and fibre cuticle cells begin to harden during the keratinization process. The inner root sheath keratinizes at a lower level of the follicle and its cuticle helps to mould the cuticle of the fibre into its final shape. The fibre and the inner root sheath move up the follicle together, with their cuticles interlocked. Approximately half way up the follicle, the inner root sheath disappears, leaving the fibre to emerge from the skin free of the inner root sheath.

In fine Merino fibres, keratinization begins in the outer part of the fibre at a lower level on one side than the other, and is termed segmental keratinization. In coarser fibres, the keratinization starts uniformly around the periphery of the fibre and proceeds towards the centre - periphero-axial keratinization [Auber (1952)]. The asymmetry in keratinization is associated with the asymmetry of the ortho- and para-cortex found in the fibre, segmental keratinization being associated with bilateral ortho-, para-asymmetry, and periphero-axial

keratinization being associated with radial ortho-, para-asymmetry.

Although our knowledge of the structure of the wool fibre has increased enormously over the past fifteen years, there are still many fundamental points, such as the structure and composition of the protofilaments, their packing and relation to the matrix protein, composition of the intrafilamentous and intrafibrillar matrix proteins, and nature and composition of the ortho- and para-cortices, which remain as yet unanswered. The isolation of pure homogeneous proteins from known sites within the fibre, and the resulting structural, sequential and conformational studies on them, will help to answer these and other questions on the detailed structure of wool and other keratin fibres.

[B] THE ORTHO-, PARA-CORTEX CONCEPT.

(i) Historical.

In 1953, Horio and Kondo discovered the bilateral structure of the cortex in fine, crimped wool fibres by showing that the cortex consists of two components differing in dye uptake [towards both acid and basic dyes] , swelling in alkali, and in birefringence on exposure to alkali. They found that the dyed component always resided on the outside of the curve of the crimp wave [when using basic dyes], and that the two components were wound around each other helically in phase with the fibre crimp. Figure 3.1 is a cross-section of wool fibres stained with gold showing the bilateral asymmetry of the cortex. Mercer (1953), during studies of enzyme digestibility and stability of fine wool fibres, also came to the conclusion that the cortex had an unsymmetrical structure. He suggested that the cortex had two components differing in chemical reactivity, which corresponded to the two components observed by Horio and Kondo (1953).

Horio and Kondo (1953) used the terms dye-accessible and non-dye-accessible to describe the two segments. Fraser and Rogers (1955a) used the terms S [soft] and H [hard], while Mercer (1953) proposed the names orthocortex and paracortex. At the 1955 Wool Conference it was decided to adopt the terms Mercer (1953) proposed; i.e. for the more easily stained, more

chemically reactive segment "orthocortex" and "paracortex" for the other segment [Proceedings International Wool Textile Research Conference, Australia 1955. F:225].

Dusenbury and Menkart (1955) point out that the bilateral structure had been on the verge of discovery for a considerable time, by a number of workers. McMurtrie (1886) and von Bergen (1935) studying the effect of alkali on wool, Woods (1935) observing changes in crimp configuration with humidity, Ohara (1938), Royer and Millson (1940) and Watkins, Royer and Millson (1944) during dyeing experiments, Ohara (1939) studying birefringence in the fibre, and Race (1946) studying fungal attack on wool, all observed phenomena which are now known to be due to the bilateral nature of the cortex.

Horio and Kondo (1953) report that Hirabayashi (1938) observed chemical differences in the two cortical components, and Mercer, Golden and Jeffries (1954) cite Schoberl (1942) as having observed asymmetric deposits of metal sulphides in fibres treated with solutions of the metal salts. Elliott and Roberts (1956) state that Zahn and Haselman (1950) observed that part of the wool cortex is more insoluble in certain reagents than the rest of the cortex. Fraser, Lindley and Rogers (1954), on reinterpreting the results of Lindley (1947), showed that he in fact separated the orthocortex [alkali soluble fraction B] from the paracortex [fraction Aa] and analysed both fractions. According to Rudall (1955a), von Bergen (1929) observed a marked difference in chemical reactivity of the two

sides of wool fibres, but attributed this to a photochemical effect. He also states that Hock, Ramsey and Harris (1941), and the prize-winning essay "the tippy dyeing of wool and its control" [Anon.(1947)], show the two sided nature of the wool fibre cortex. In 1936, Rudall showed that keratinization of the concave side of the crimp wave was completed more rapidly than the convex side. Also Freney (1947), and later Goldsworthy and Lang (1953), observed coiling of fibres in various liquids, which is now known to be due to the bilateral asymmetry within the fibre.

Prior to 1953, the results mentioned above were probably thought to be due to the variability of the material, or due to some form of freak damage of the fibre and therefore not given any prominence. The work of Horio and Kondo (1953) and Mercer (1953) started the extensive research into the chemical and physical nature of the ortho- and para-cortices.

(ii) Dyes, Metals and Colour Reactions Which Produce Differentiation.

Horio and Kondo (1953) repeated the experiments reported by Ohara (1938) using his technique and dyes Janus Green B [C.I.* part 2, No. 11050], a basic dye, and the acid dye Orange G [C.I. acid orange 10]. However, they found that the acid dye Ponceau 2R [C.I. acid red 26] gave better results than Orange G and used it in preference. Janus Green B has also been

*C.I. - Colour Index, second edition (1956) and supplement (1963) [Published by Society of Dyers and Colourists, Bradford, England].

used by Mercer (1954d), Auber and Ryder (1955), Dusenbury and Coe (1955), Dusenbury and Jeffries (1955), Dusenbury and Menkart (1955) and Menkart and Coe (1958) to study the ortho-, para-cortical distribution in fibres.

When using the acid dyes Orange G and Ponceau 2R, Horio and Kondo (1953) found that the portion of the fibre dyed always resided on the inside of the crimp wave, the reverse of staining with basic dyes, and concluded that the para-cortex was dyed. Dusenbury and Coe (1955) have since shown conclusively that this is incorrect and that the acid dyes Ponceau 2R and Formyl Violet S4B [C.I. acid red 17] preferentially stain the orthocortex, as does the basic dyes. This was substantiated by Menkart and Coe (1958) using the acid dyes Formyl Violet S4B, Violamine R [C.I. acid violet 9] and Indocyanine B [C.I. acid blue 98]. Both authors suggested that Horio and Kondo (1953) used dyeing conditions which resulted in a reversal of crimp and so led to their anomalous result.

Horio et al. (1960) have concluded that basic dyes stain the orthocortex, but "true" acid dyes [i.e. dyes which only contain sulphonic acid groups] stain the paracortex. Acid dyes which contain basic groups, as well as sulphonic acid groups, stain the orthocortex - the basic groups apparently "overwhelm" the acid groups. They also state that direct dyes and chrome dyes stain the paracortex. If one correlates the structure of the dye with the segment stained, according to the scheme above, then all observed dyeing results and anomalies can be explained.

The most commonly used dye to investigate the cortical asymmetry of wool has been Methylene Blue [C.I. basic blue 9], which dyes the orthocortex blue and leaves the paracortex unstained [Mercer (1954a), Fraser, Lindley and Rogers (1954), Fraser and Rogers (1955a,c), Fraser and MacRae (1956), Elliott and Roberts (1956), Ahmad and Lang (1956, 1957), Haly (1957), Lang (1958), Menkart and Coe (1958), Elliott, Asquith and Rawson (1958a,b, 1959), Ryder (1959), Leveau (1959b), Rogers (1959a,b), Snaith (1960), Jones (1961, 1966a),Williams (1962), Andrews, Feughelman and Mitchell (1962), Baird (1962, 1963), Haly (1963), Davies (1963a,b, 1965), Snyman (1963a,b), Haly and Inglis (1964), Shah and Whiteley (1966), Suarez (1966)]. Various dyeing conditions [time, temperature, pH and concentration of dye] have been used by the above authors, with no agreement on the optimum dyeing conditions to achieve bilateral staining.

Fraser and Rogers (1954, 1955a,c) and Fraser, Lindley and Rogers (1954) have found that pretreatment with peracetic acid prior to staining with Methylene Blue improves the differentiation but the staining is reversed. The dyeing is metachromatic, the colour of the paracortex being purplish and that of the orthocortex the usual blue. Menkart and Coe (1958) have also observed the reversal in dyeing after a pretreatment with either peracetic acid or bromine water and also found that after short exposure to the dye solution, the dye in the paracortex can be readily rinsed away. Clarke and Maddocks (1965) and Chapman (1965) have used Methylene Blue after a performic acid

pretreatment and then counterstained with Eosin [C.I. acid red 87], with the result that the paracortex stains blue, the orthocortex pink and the medulla, if present, stains red. Fraser and Rogers (1955a) dyeing wool roots, used a similar procedure except that prior to counterstaining with Eosin they stained with Mayer's Haemulum. Dyeing with Toluidine Blue [C.I. basic blue 17], after an oxidative pretreatment, also stains the paracortex [Fraser and Rogers (1955a)], which again is the reverse of staining wool with no prior oxidative pretreatment [Fraser, Lindley and Rogers (1954)].

Other dyes have been used for demonstrating the two component structure of the cortex of wool, and include Coomassie Violet [C.I. acid violet 49] [Haly (1957), Lang (1958)]; Methyl Violet [C.I. basic violet 1], Basic Fuschin [C.I. basic violet 14], Safranin [C.I. basic red 2] and Ponceau S [C.I. acid red 9] [Spearman and Barnicot (1960)]; Chicago Blue 6B [C.I. direct blue 1] followed by treatment in 1% sodium isopropionate in isopropyl alcohol, initially stains the orthocortex violet and the paracortex blue, but after 24 hours the orthocortex loses its colour [Ritter and Tomopulos (1959), Ritter and Reumuth (1960)]; Night Blue R [C.I. basic blue 6], Procion Brilliant Red 2BS [C.I. reactive red 1] [Ritter and Reumuth (1960)]; Acid Violet 3BN Extra [C.I. part 2 No. 42620], Alkali Violet 6B [C.I. part 2 No. 42630], Xylene Cyanole FF [C.I. acid blue 147], Crystal Ponceau [C.I. acid red 44] and Orange II [C.I. acid orange 7] [Horio et al.(1960)];

and the dyes Rhodamine B Extra [C.I. basic violet 10] and Nile Blue [C.I. basic blue 12] [Suarez (1966)].

Several fluorescent stains have also been shown to dye wool bilaterally. Spearman and Barnicot (1960) show that the orthocortex of wool dyed with Acridine Orange GN [C.I. acid orange 12], after a pretreatment with trypsin, fluoresced orange. Appleyard and Lees (1965) have used several fluorescent dyes. In untreated and alkali treated wool, stained with Acridine Orange [C.I. basic orange 14], the orthocortex fluoresced yellow and the paracortex green. With Rhodamine 3GO [C.I. basic red 3], on alkali treated fibres, the orthocortex fluoresced orange or red and the paracortex yellow or orange. Using Uranin [C.I. acid yellow 73], the orthocortex fluoresced a darker green than the paracortex in both untreated and alkali treated fibres. Fibres treated with hydrogen peroxide and stained with the mixed dye Geranine G [C.I. direct red 48] and Thioflavine [C.I. basic yellow 1] showed brown fluorescence in the orthocortex and green in the paracortex.

Several reactions yielding coloured products which are asymmetrically distributed, have also been described. After reaction of wool with ninhydrin at pH 5.2, the paracortex is coloured more than the orthocortex [Williams (1962)]. He also states that after the Pauly reaction [i.e. treatment of wool in the dark with Brentamine Fast Orange GR Salt (C.I. azoic diazo component 6)], when carried out on wool which is weathered or has had an alkaline or oxidative pretreatment,

stains the orthocortex more than the paracortex. Segmentation is also revealed by reaction with Schiff's reagent [bis-N-aminosulphinic acid which is formed by the reaction of Basic Fuschin with sulphurous acid], after a pretreatment with peracetic acid [Fraser and Rogers (1954)].

There have been many reports of metals asymmetrically distributed in the cortex of fibres stained with solutions of their salts. Laxer and Ross (1954) showed that the paracortex was stained red in wool fibres preswollen in formic acid and dyed with gold chloride. Mercer, Golden and Jeffries (1954), Thorsen (1958) and Priestley (1966), using sodium plumbite solutions, have shown the paracortex is stained darker than the orthocortex.

Mercury and mercuric salts have been used to stain the cortex bilaterally. Mercer (1954e) used mercuric carroting liquids and found they stained the orthocortex reddish. Menkart and Coe (1958) showed that in cross-sections of wool fibres, exposed to mercury vapour, the paracortex stained more heavily than the orthocortex. Kassenbeck (1965b) has also stained the paracortex using ammoniacal solutions of mercury salts.

Corbett and Yu (1964) have studied the conditions of bilaterally dyeing the orthocortex of wool with several metal ions in acidic nitrite solution. Chromic ions give a bright yellow, cobaltous [olive green], cupric [dull purple], ferric [yellow], manganous [greenish yellow] and nickelous [pinkish brown]. Mercer (1954e), Thorsen (1958) and Priestley (1966)

have also used acidic nickelous nitrite solutions to stain wool bilaterally, and Lang and Campbell (1966) acidic cupric nitrite solutions.

When cross-sections of wool fibres, stained with metals, are viewed in the electron microscope, the ortho- and paracortices can be distinguished. Rogers (1959a,b), Filshie and Rogers (1961) and Leach, Rogers and Filshie (1964) have used osmium tetroxide after reduction with thioglycollic acid, Kassenbeck (1965a,b), Kassenbeck and Hagege (1965), Nott and Sikorski (1965) and Derminot, Tasdhomme and Parisot (1965) have reported using silver acetate or nitrate after reduction with thioglycollic acid, and Derminot, Tasdhomme and Parisot (1965) and Kassenbeck, Jaquemart and Monrocq (1965) have used thallium carbonate to observe ortho-, para-cortical staining.

Electron microscopic and light microscopic studies of asymmetrically stained wool fibres, show that the ortho- and para-cortical segments consist of two clearly defined sets of cortical cells, with the boundary between them following the cortical cell outlines i.e. the boundary runs along the cortical cell membranes. A cortical cell is either ortho or para in structure; no cortical cell contains some ortho and some para characteristics.

Other methods of observing and demonstrating the ortho- and para-cortices in wool have been reported. Bromine colours the orthocortex bright yellow [Thorsen (1958)] and in some pigmented fibres melanin has been observed to be asymmetrically

distributed in the cortex [Laxer, Whewell and Woods (1954), Laxer and Whewell (1955), Haly (1957)]. Haly (1957) has shown that the ortho- and para-cortices can be distinguished in fibres saturated with water, mounted in paraffin oil and observed in the light microscope. Dusenbury (1960), in an excellent paper, shows that the type of asymmetry can be deduced from the solubility of keratins in carefully prepared urea-bisulphite solutions.

The difference in birefringence of the ortho- and para-cortices, when wool fibres are immersed in NaOH solution, has been used by many workers to distinguish between the cortices. Ohara (1939) was probably the first to observe this phenomenon, but it was Horio and Kondo (1953) who showed that the birefringence of the orthocortex disappears, while that of the paracortex does not alter after the caustic solution is applied to the fibre. Others that have used this method are Fraser and Rogers (1953, 1955a), Mercer (1953), Dusenbury, Mercer and Wakelin (1954), Fraser and Rogers (1954), Fraser, Lindley and Rogers (1954), Mercer (1954c,e), Mercer, Golden and Jeffries (1954), Dusenbury and Coe (1955), Dusenbury and Jeffries (1955), Dusenbury and Menkart (1955), Golden, Whitwell and Mercer (1955), Menkart and Coe (1958), Satlow and Kessler (1958), Satlow (1959), Dusenbury (1960), Louw (1960b) and Horio et al. (1965).

Mercer (1954a) found that when a suspension of cortical cells, isolated by a trypsin treatment, was dyed with Janus Green B [C.I. part 2 No. 11050], about half of the cells were stained

more deeply. However he does not indicate which cells are stained the deepest, or whether they are indeed related to the ortho- and para-cortical cells.

Leveau (1956a) has reported that Lactophenol Cotton Blue [a 1% solution of Cotton Blue (C.I. acid blue 93) in Lactophenol - a mixture of lactic acid, phenol and glycerine in water] stains the orthocortex of wool, after the cuticle has been damaged. He also states that cortical cells isolated by an acid treatment can be differentially stained with this dye.

(iii) Differentiation in Various Keratin Fibres.

The differentiation of a wool fibre into two components bilaterally arranged in the fibre, is an oversimplification. It is true for the finer crimped wool fibres, but not for coarser wool fibres and other keratin fibres.

Ahmad and Lang (1956) have observed nine different patterns of differentiation in Pakistani Carpet wools, and later, (1957), they observed similar patterns of differentiation in "anomolous" or "doggy" wool. Their results, in agreement with those of Fraser and Rogers (1955a,c), also show that the pattern of differentiation is a function of diameter. The fine fibres have bilateral asymmetry and the coarser fibres have the ortho- and para-cortex radially distributed.

Fraser and MacRae (1956) report that crimpless mutant Merino wool has a central core of orthocortex surrounded by paracortex, which has been confirmed by Haly (1957) and by Menkart and Coe (1958).

Dusenbury and Jeffries (1955) and Dusenbury and Menkart (1955) have found that Buenos Aires fleece wool had ortho- and para-cortical cells, which were later shown to be radially distributed [Menkart and Coe (1958), Dusenbury (1960)]. In copper deficient or "steely" wool, Fraser and Rogers (1955a) found that the bilateral differentiation was not as distinct as in normal wool. This was confirmed by Haly (1957), Louw (1960 b) and Nott and Sikorski (1965). Hardwick and Romney wool show bilateral differentiation [Auber and Ryder (1955)], as also does Rambouillet 64's wool [Dusenbury and Menkart (1955)]. Burmese wools in general show radial differentiation, but in the coarser fibres [60-140μ] no differentiation could be seen [Lang (1958)]. Corriedale wool has bilateral asymmetry [Fraser and Rogers 1955a,c)], although some fibres have radial asymmetry [Haly (1963)]. Haly and Griffith (1958) state that the finer Corriedale fibres have bilateral asymmetry and the coarser fibres radial asymmetry.

Lincoln wool has been shown to have radial asymmetry, with the paracortex surrounding the orthocortex [Fraser and Rogers (1955a,c), Fraser and MacRae (1956), Dusenbury (1960), Williams (1962), Haly (1963)]. Shah and Whiteley (1966) have also shown that Lincoln fibres have the ortho- and para-cortices radially distributed. They state that in most Lincoln wool fibres, the paracortex surrounds the orthocortex, but in some fibres the cortices are reversed, with the paracortex in the centre surrounded by orthocortex.

Laxer, Whewell and Woods (1954) demonstrated that the pigment in several "crimpy" wools [Welsh Mountain, Portugese Seragosa, Yorkshire-grown Leicester, Leicester-Dalesbred] was mainly concentrated in the paracortex. Their findings were extended to show that the melanocytes producing the melanin granules [pigment] were asymmetrically distributed in the follicle [Laxer and Whewell (1955)]. Haly (1957) confirmed their findings in lightly pigmented Merino wool fibres, but could not observe differentiation in highly pigmented fibres.

Dusenbury and Jeffries (1955) and Dusenbury and Menkart (1955) concluded that Human hair consisted entirely of paracortical cells, and Mohair or orthocortical cells. These conclusions were further supported by evidence obtained by Menkart and Coe (1958) and Haly and Griffith (1958).

Spearman and Barnicot (1960) have examined spiralized Negro and Bushman hair [both of African origin]. They found that no bilateral differentiation could be detected by dyeing studies, but the melanin was asymmetrically distributed, the greater concentration being on the concave side of the curl. Mercer (1954b), however, has stated that Negro hair has ortho- and para-cortical cells bilaterally distributed as shown by a stability test.

The observations that Mohair consists entirely of orthocortical cells must be treated with some doubt, as it has been shown that Mohair can be dyed radially, with the paracortex surrounding a dyed central core of orthocortex [Fraser and MacRae

(1956), Thorsen (1958), Appleyard and Perkin (1963), Suarez (1966)]. Fraser and MacRae (1956) reinvestigated the Kid Mohair samples from which Dusenbury and Menkart (1955) based their conclusions that Mohair contains only orthocortex, and found that they did show radial ortho- and para-cortical dyeing, but not as clearly as did adult Mohair. They also suggested that the discrepancy was due to a poor choice of dye by Dusenbury and Menkart for examining ortho-, para-cortical effects.

The curves for alkali solubility that Dusenbury and Jeffries (1955) and Dusenbury and Menkart (1955) report as evidence that Mohair only contains orthocortical cells, can also be interpreted as indicating that there is some paracortex present in the structure [approximately 9%]. Dusenbury (1960) found from urea-bisulphite solubility studies, that Mohair contains mainly orthocortex, but has some paracortex.

Cashmere and Vicuna fibres have bilateral cortical asymmetry [Dusenbury (1960)]. Menkart and Coe (1958), in a footnote, also state that Cashmere has bilateral asymmetry. The pigment in Vicuna is distributed bilaterally, the paracortex being more heavily pigmented than the orthocortex [Laxer, Whewell and Woods (1954)].

Two different species of Alpaca, however, showed different asymmetry. Huacayo Alpaca [crimped] shows bilateral asymmetry whereas Suri Alpaca [relatively straight] exhibits radial asymmetry [Dusenbury (1960)].

Auber and Ryder (1955) report that Cattle tail hair

[a straight fibre] has radial asymmetry, whereas fibres from the Sloth, which have a regular undulation comparable to crimp, the asymmetry is bilateral. Mercer (1954e) suggests that Rabbit fur may have an incipient bilateral asymmetry of the cortex, but this has not been definitely proved.

In an interesting note, Priestley (1966) shows that adult albino Rat hair, albino Mouse hair, Dog underhair [adult Border Collie], Red Deer underhair, Mouflon wool and wool from a Kerry Hill ewe lamb all show bilateral asymmetry. With the zig-zag hair from Mouse and Rat, the bilateral asymmetry only occurs at the bends in the fibres and it is reversed to that observed in wool i.e. the paracortex is on the outside of the crimp wave. The asymmetry observed in the Mouflon wool shows that the bilateral differentiation was present in wild sheep and indicates that the bilateral differentiation is not an artefact of domestication of the sheep.

Blakey et al. (1965), investigating the vibrissae [whiskers] of Lion, found that the outer part of the cortex resembled orthocortical cells and the inner part, paracortical cells i.e. showed radial asymmetry. Kidd (1965) reports that no ortho-, para-cortical differentiation was obtained when the coarse hairs, used for brush making, were dyed with methylene blue.

The above results indicate that, in general, for crimped keratin fibres the cortex will have bilateral asymmetry, and straight fibres will have radial asymmetry. However, each type of keratin fibre should be investigated separately and carefully

to distinguish the exact nature of the differentiation, if any.

Several methods for measuring the percentage of the two cortices in cross-sections of dyed fibres have been used. Three methods have been compared by Davies (1965) and two others by Jones (1966a).

The percentage paracortex in Merino wool is about 37%, with a range from about 32% to 46%. Ahmad and Lang (1957) give a mean value of 37.3%; Jones (1961). 39.3%; Davies (1963b), 34.7%; Chapman (1965), 45.2% and 38.5% on the same sample of wool using two different dyeing methods; and Jones (1966b), 38.3%. Thorsen (1958) shows that the percentage paracortex increases with increase in fibre diameter, in agreement with Ahmad and Lang (1957).

Ahmad and Lang (1956) have shown that the percentage paracortex is higher in medullated Pakistani wools [53.6%] than in nonmedullated [49.7%]. Later (1957), they showed that "anomalous" or "doggy" Merino wool had a greater percentage paracortex than normal Merino wool [55% against 37.3%]. Similar results were obtained by Jones (1961) and Chapman (1965). The level of nutrition does not affect the amount of paracortex, the percentage being the same for wool from sheep on a low [starvation] feeding level and wool from sheep on a high feeding level [Slinger, Smuts and Mellet (1966)].

Davies (1963a,b) shows that in cross-sections, the depth of staining with methylene blue decreases on exposure to light, but increases again if the slide is stored in the dark. However,

the fading of the stain has no effect on either the precision or the accuracy of the paracortex estimation.

Recently, Jones (1966a) completed an important investigation in which he determined the percentage paracortex at different levels of several wool fibres. The percentage of paracortex varies over large limits e.g. fibre No. 7 [from a sample of pen grown Merino wool] varied from 14.6% to 60.1%, with a mean of 41.7%. More surprising, the pattern of differentiation also varies considerably along the length of the fibre. The ortho-, para-cortical differentiation of fibre No. 7, at various levels, is reproduced in Figure 1.8.

mm 44.045 88.0 88.035 110.01 110.025

Figure 1.8. Pattern of Differentiation along a single Merino fibre, No. 7, from Jones (1966). (Distances in mm from 1st section).

He has also found that for fibre No. 5, the minimum percentage of paracortex [19.9%] and the maximum percentage of paracortex [44.8%] were obtained in sections 20 microns apart. This large change, over a distance considerably less than the length of a cortical cell, is difficult to explain. The author suggests that either the staining method [using Methylene Blue] is inadequate, or that changes occur along the length of cortical cells. The latter seems unlikely, as cortical cells are either ortho or para in structure. Nott and Sikorski (1965),

during an electron microscopic investigation of birthcoat fibres from sheep, have also observed that the amount of paracortex can vary enormously and that these fine structural variations cause differences in external form.

(iv) Chemical and Physical Differences of the Two Cortices.

The ortho- and para-cortices react differently to various chemical reagents. In general the orthocortex is the first to be attacked, having a higher chemical reactivity than the paracortex.

Apart from the previously mentioned change in birefringence in the orthocortex when wool is immersed in alkali, it has been shown that aqueous and ethanolic alkali preferentially attacks and swells or completely dissolves the orthocortex [Horio and Kondo (1953), Dusenbury and Coe (1955), Dusenbury and Jeffries (1955), Fraser and Rogers (1955a), Elliott and Roberts (1956), Leveau (1956b), Haly (1957), Elliott, Asquith and Rawson (1959), Leveau (1959a) Daveloose, Mazinque and Van Overbeke (1960), Louw (1960b)]. Due to the higher swelling of the orthocortex compared to the paracortex, wool fibres tend to coil when immersed in alkali solutions. Freney (1947) observed this phenomena but was unable to give any explanation of it. Goldsworthy and Lang (1953) suggested it was due to relaxation of torsional "cohesive set". Dried wool roots also show differential swelling on exposure to alkali and detergents [Fraser and Rogers (1954)].

Wool pretreated by boiling in sulphuric acid, dissolves in alkali in two stages, the orthocortex dissolving at a faster

rate than the paracortex. Dusenbury, Mercer and Wakelin (1954) and Dusenbury and Menkart (1955) have derived a first order rate equation for the dissolution process, showing that the orthocortex dissolves about eleven times faster than the paracortex [0.477 $hour^{-1}$ and 0.0441 $hour^{-1}$ respectively]. However in another paper by Dusenbury and Jeffries (1955), the rates, for the same wool and apparently using the same conditions, are quoted as 0.263 $hour^{-1}$ and 0.0386 $hour^{-1}$ respectively.

Leveau (1959a) found that the orthocortex in wool, and separated orthocortical cells, were more readily dissolved in 4N HCl [1 hour, 65°C] than the paracortex or paracortical cells respectively. Elliott and Roberts (1956) reported that the orthocortex is dissolved when wool is boiled in either 1N or 2N HCl, leaving the paracortex. Eventually, on prolonged treatments, the paracortex is also dissolved. Dilute HCl and oxalic acid, in the presence of secondary alcohol sulphate detergents, dissolves the orthocortex but does not attack the paracortex [Fraser, Lindley and Rogers (1954)].

Elliott, Asquith and Rawson (1959), studying the action of sulphuric acid on wool, found that the orthocortex is attacked and dissolved before the paracortex in both 47% and 98% H_2SO_4.

Mercer (1953) found that after oxidation by peracetic acid, wool fibres showed bilateral asymmetry when treated with dilute ammonia [0.1N]. Fraser and Rogers (1955a) re-examined the above phenomenon and observed that the orthocortex swells more than the

paracortex - in fact the swelling was enough to burst the cuticle on the orthocortex side of the fibre. However, if after oxidation with peracetic acid the fibre is immersed in water, then the paracortex swells more than the orthocortex [Menkart and Coe (1958)].

Alkaline thioglycollate [pH 12.3] has been shown to attack the orthocortex preferentially [Fraser and Rogers (1953, 1955b)]. Blisters on the cuticle appear around the orthocortex side of the fibre [Fraser and Rogers (1955a)] and under carefully controlled conditions, the orthocortex can be preferentially extracted [Fraser and Rogers (1955c)].

Treatment of wool with Cadoxen [cadmium triethylenediamine hydroxide], first destroys the birefringence in the orthocortex and then in the paracortex. Immersion of the treated wool in 5N NaOH returns the birefringence in the paracortex [Suarez (1966)]. After 45 minutes the fibre swells about 200% in the Cadoxen.

Cupriethylenediamine preferentially dissolves the orthocortex [Leveau (1959a,b), Daveloose, Mazinque and Van Overbeke (1960)], as does formamide [Leveau (1959a)] and concentrated sodium sulphide solutions [Daveloose, Mazinque and Van Overbeke (1960)]. In glacial acetic acid, the orthocortex swells rapidly and loses its birefringence, whereas there is no alteration in the paracortex [Fraser and Rogers (1955a)]. The bilateral structure can also be rendered visible by treatment with LiBr in methanol [Ritter, Reumuth and Tomopulos (1960)].

Leveau, Cebe and Parisot (1953) suggested that the cuticle on the orthocortex side of wool fibres must be different to that on the paracortex side of the fibre. This conclusion was reached after observing that Allworden bubbles [produced by the action of fresh bromine water on wool] were only formed on the orthocortex side of the fibre. Fraser and Rogers (1955d) have since shown that this is incorrect and that the Allworden bubbles appear on the orthocortex side first and then spread all around the fibre surface. Leveau (1959a) and Menkart and Coe (1958) have shown that bromine water preferentially attacks and dissolves the paracortex, which is the only chemical treatment reported in the literature that preferentially attacks the paracortex.

The orthocortex can be extracted with bisulphite [5% sodium metabisulphite] in saturated urea solutions [24 hours at 40°C], leaving a residue consisting of paracortex, orthocortical cell membranes and the resistant cuticular sheath [Mercer (1954c)]. The orthocortex of wool, treated with urea-bisulphite solutions at 65°C, loses its birefringence rapidly. However a pretreatment with sodium carbonate causes the loss in birefringence to take 3 - 4 times longer [Satlow and Kessler (1958)]. Dusenbury (1960) has used the solubility in urea-bisulphite as a test for determining the type of ortho- para-asymmetry in keratin fibres. His results agree well with those of other workers using dyeing techniques.

Leveau (1959a) has tested the solubility of isolated

ortho- and para-cortical cells in urea-bisulphite solution and found that they dissolve to the same extent. If the isolated cells are first heated, the solubility of the paracortical cells remains the same, but that of the orthocortical cells decreases. He has also observed similar results in the solubility [in urea-bisulphite] of iodinated cortical cells, the orthocortical cells becoming almost insoluble [Leveau (1959d)].

In wool treated with saturated aqueous urea solutions at 65°C for several days, the orthocortex swells appreciably, loses its birefringence and appears similar to wool treated with 0.1N NaOH. The addition of a secondary alcohol sulphate detergent to the urea solution accelerates the swelling, the orthocortex swelling to about eight times its original volume. Treatment of these highly swollen fibres with glass balls in a mechanical agitator gives cuticle, paracortical cells and a jelly-like mass from the orthocortex [Fraser and Rogers (1955a)].

Enzymes preferentially attack and dissolve the orthocortex in wool [Mercer (1954a,d), Fraser and Rogers (1953, 1955a)], as do fungi [Fraser and Rogers (1955a)] and clothes moth larvae [Tineola bisselliella] [Mercer (1953)]. If feeding rapidly, the clothes moth larvae will only digest the ortho-cortex and reject the paracortex and cuticle as waste.

When wool is supercontracted [in many liquids], it is found that the orthocortex supercontracts more than the paracortex. This generally leads to a reversal of the distribution of the two cortices in the crimp wave - the

orthocortex [after supercontraction of the fibre] occupying the inside of the curvature, e.g. with lithium bromide [Haly (1957)], the orthocortex supercontracting 16% and the paracortex 4.5% [Haly and Griffiths (1958)]. A short treatment with urea-bisulphite solution causes the orthocortex to supercontract, but immersion in this solution for a long time also causes the paracortex to supercontract and then the fibre straightens [Mercer (1954c)].

Mercer (1953) has shown that the orthocortex supercontracts when wool is heated in water at 120°C, heated in formamide or heated in aqueous phenol. Leveau (1956a) obtained differential dyeing of isolated cortical cells in Lactophenol Cotton Blue. He states that the phenol in the dye provokes a longitudinal contraction, together with a lateral swelling, of some cells [which he identifies with orthocortical cells] and not in others [paracortical cells].

Haly (1963) has investigated the supercontraction of fibres which have radial asymmetry of the two cortices [Lincoln and Corriedale wool] in concentrated lithium bromide solutions. After grinding away the paracortex, he found that the remaining orthocortex supercontracted to a greater extent than the whole fibre, and thus concluded that the paracortex [in these fibres] determines the overall fibre supercontraction.

The rate of sorption in the two cortices is different, being faster in the paracortex than in the orthocortex [Williams (1962)]. The greater degree of swelling in the orthocortex has been suggested as the cause of the entanglement of wool fibres,

causing wool to felt [Dobozy (1958)]. Baird (1963) has shown that a fibre with bilateral asymmetry acts like a bimetallic strip during swelling. The change of arc radius of a Merino wool fibre set in a curve is about four times that of a Human hair fibre [which is considered to have no asymmetry of its cortex, being pure paracortex in nature].

The ortho- and para-cortices behave differently when heated in air, vacuum, nitrogen or water [Leveau (1959c), Daveloose, Mazinque and Van Ovebeke (1960), Horio et al. (1965)].

Fraser and Rogers (1955a) reported that preliminary experiments indicated that the paracortex is twice as hard as the orthocortex. Feughelman and Haly (1960) studied the mechanical properties [load extension curves in distilled water and in HCl at pH 1] of Lincoln fibres, and orthocortex from these fibres, obtained by grinding away the paracortex. They found that the fibre was stiffer than the [abraded] orthocortex, implying that the paracortex is stiffer than the orthocortex. They concluded this was due to the paracortex containing numerous salt linkages, whereas the orthocortex contains none. The ortho- and para-cortex have the same rigidity when dry, but when wet the rigidity of the orthocortex is three times lower than that of the paracortex [Andrews, Feughelman and Mitchell (1962)]. The above measured mechanical properties all indicate that the paracortex is stronger than the orthocortex, which is in agreement with the results of Chapman (1965) who shows that as the percentage of paracortex increases, the strength of the fibre also increases.

The isoelectric point of the two cortices has been determined histochemically by Horio et al. (1960). They found that the value for the orthocortex of Merino wool was 5.90, and for the paracortex, 6.75. For Corriedale wool the values were 6.15 and 6.90 respectively.

(v) Separation of Ortho- and Para-cortices of Wool.

The isolation of either the paracortex or ortho- and para-cortical cells from wool has been accomplished by three methods - (i) chemical (ii) combination of chemical and enzymatic and (iii) mechanical. All three methods have, however, been open to criticism on several grounds. Either the method does not produce a pure component, or in some cases two components have been isolated, but which component came from the orthocortex and which came from the paracortex could not be determined. In the methods based on chemical or enzymatic separation there is an unknown amount of damage to one or both components. With the mechanical methods reported so far, there is some likelihood of modification of the structure due to local or overall heating during the treatment. Thus all analyses reported to date on isolated fractions must be viewed with considerable conservatism.

Probably the first separation, and certainly the first analysis of the ortho- and para-cortex, were completed before the existence of the two cortices was discovered by Horio and Kondo (1953). Fraser, Lindley and Rogers (1953) showed that Lindley (1947) had dissolved the orthocortex of wool in dilute alkali following a partial hydrolysis with cetyl-sulphonic acid

[0.05M, for 6 days at 65°C], leaving a residue which consisted largely of paracortex. He had (1947) analysed both residue and dissolved fraction [orthocortex].

There have been several methods developed for the removal of the orthocortex by enzymes from wool given various pretreatments. Fraser and Rogers (1955a) used papain in the presence of urea-bisulphite which degrades the orthocortex. The paracortical cells can be recovered by shaking the fibres with glass balls and pouring through a 200 micron mesh sieve, the disintegrated orthocortical cells passing through and leaving the paracortical cells behind. Mercer, Golden and Jeffries (1954) obtained the paracortex of wool either by (i) using thioglycollic acid [pH 4.5 for 8 hours] which reduces the disulphide bonds, and then alkylated the free-SH groups with ethyl bromide; the orthocortex was then digested with pepsin or (ii) the orthocortex was supercontracted by treatment with urea-bisulphite solution, by heating in phenol or in formamide and then digesting with trypsin to give a residue of paracortex plus cuticle.

The commonly used enzyme treatment for observation of ortho- para-cortical effects, or for removal of the orthocortex, has been one developed by Mercer (1953). This involves supercontracting the orthocortex by treatment in water at 120 - 130°C for 1-1/2 to 4 hours and then digesting the orthocortex with trypsin. Others who have used this method are Laxer, Whewell and Woods (1954), Mercer, Golden and Jeffries (1954),

Dusenbury and Jeffries (1955), Dusenbury and Menkart (1955), Golden, Whitwell and Mercer (1955), Laxer and Whewell (1955), Lockart (1960).

Using the above method Lockhart (1960) found that the lengths of cortical cells, freed from Merino wool, do not show a difference between paracortical cells and the overall mean of ortho- plus para-cortical cells from the same fibre.

The most commonly used method for obtaining ortho- and para-cortical cells is based on partial hydrolysis of the wool fibre with HCl, followed by separation of the liberated cortical cells. Ward and Bartulovich (1955) first described the partial acid hydrolysis method which was later used by Lundgren (1955), Ward and Bartulovich (1956), and Simmonds and Bartulovich (1958). These workers treated wool in 6N HCl at 26°C for 48 - 488 hours and then mechanically disintegrated the fibres. The separation of the cortical cells into two fractions was accomplished by using chloral hydrate density gradients. The lighter cortical cells [density, 1.499 gm cm $^{-3}$] had a lower sulphur content [3.53%] than the heavy cortical cells [density, 1.512 gm cm $^{-3}$ and sulphur content 5.20%]. During the HCl treatment, 30 - 40% of the wool was dissolved. Also the identity of each fraction was not established. Later, Ward and Bartulovich (1964) and Bartulovich (1964) report that they were able to mechanically disintegrate wool in a ball mill cooled to liquid nitrogen temperature without any change in the nitrogen or sulphur content, or infra-red spectrum. The disintegrated wool can again be

separated into two fractions on chloral hydrate gradients.

Leveau (1956a,b) modified the method that Ward and Bartulovich (1955) used to obtain cortical cells. He performed the partial hydrolysis with 6N HCl, but used shorter times at higher temperatures. After mechanically disintegrating the partially hydrolysed fibres, the ortho- and para-cortical cells can be separated by a sieving process [Leveau (1956b)].

Several French and Spanish workers have used the method above to obtain separated ortho- and para-cortical cells and include Derminot and Leveau (1956), Leveau (1957, 1958, 1959a, c,d,e), Derminot (1958), Daveloose, Mazinque and Van Overbeke (1960), Miro (1961), Miro and Blade (1965), and Parisot, Allard and Baures (1965).

Leach, Rogers and Filshie (1964) have dissolved the orthocortex with boiling HCl [pH 2, for 96 hours], leaving undissolved the paracortex and exocuticle, apparently unmodified as far as can be judged by electron microscopy.

Horio et al. (1965) found that when wool is heated at 170°C in the presence of water for several hours, the fibre splits along the ortho- para-cortical boundary. After the heat treatment, all the wool can be split into the two separate cortices by grinding in a mortar and pestle. The two cortices can then be separated by means of a chloral hydrate density gradient. They identified the layers by a dyeing method [which is not very clearly stated in the paper] and claim that the paracortex has a density of 1.3806 gm cm^{-3} and the orthocortex a density

of 1.2274 gm cm^{-3}. These values of density are in marked contrast to the values Ward and Bartulovich report for heavy [1.512 gm cm^{-3}] and light [1.499 gm cm^{-3}] cortical cells obtained from wool by a partial acid hydrolysis [see above]. This indicates that either or both sets of cortical cells are chemically modified, so that the uptake of chloral hydrate is different in the two cases giving the vastly differing density values.

Haly (1960) has developed a method for uniformally abrading wool fibres over 1 cm lengths. In the case of fibres showing radial asymmetry of the two cortices [e.g. Lincoln and Corriedale wool], the outermost segment [paracortex in general] can be ground away to leave only the orthocortex. By this means Haly and Inglis (1964) obtained enough orthocortex from Lincoln wool fibres to perform amino acid analyses. Snaith (1960) has improved the apparatus so that 10 cm fibre lengths can be uniformally abraded to leave only the orthocortex.

The composition of the two cortices isolated by the above means, and obtained by other methods, will be discussed in detail in section 3.

(vi) The Origin of the Two Cortices and Their Relation to Crimp.

Originally, when it was first recognized that two cortical segments of different reactivity and dyeing behaviour existed in the cortex, it was thought that the difference was due to asymmetric keratinization [Mercer (1954a,b)]. The observations of Rudall (1936), Auber (1952), Fraser and Rogers (1954), Auber and Ryder (1955) and Horio et al. (1960) showing that the

paracortex [in fibres with bilateral asymmetry] was keratinized first, supported the above view. However, it has been shown that cortical cells before keratinization, show segmentation [Fraser and Rogers (1954, 1955a,c)]. Laxer, Whewell and Woods (1954) and Laxer and Whewell (1955) also showed that melanin granules [or pigment] are asymmetrically distributed in the prekeratinized region of the cortex. Fraser (1964) suggests that cells maintaining mitotic activity give rise to paracortical cells, whereas those cells differentiating give rise to the orthocortex.

Fraser and Rogers (1955b) suggested that the bilateral segmentation of the fibre cortex occurred at the germinal level of the bulb, but could not substantiate this. Fraser's (1964) results support this contention, but do not conclusively prove it. Keratinization must not be ruled out altogether as a contributing cause of the differences observed in the two cortical components. It may exaggerate the differences that already exist before keratinization.

The weight of evidence supports the view that changes occurring in the two cortical components during keratinization , give rise to the characteristic crimp of bilaterally asymmetric fibres. Dobozy (1959) suggests that crimp is due to the asymmetrical swelling of the two cortices, the orthocortex swelling and the paracortex shrinking [supercontracting] during keratinization. This is supported by Louw (1960b), who points out that "steely" or copper-deficient wool is uncrimped but has bilateral asymmetry. "Steely" wool, on emergence from the

follicle, is incompletely keratinized [due to lack of copper which acts as a catalyst], keratinization continuing in the fibre for six to eight months. Thus the asymmetric swelling of the two cortices has not taken place and the fibre remains straight. Kassenbeck (1965a) also concludes that crimp appears to result from supercontraction of the paracortex during keratinization.

In marked contrast to the above view on the formation of crimp, Chapman (1964, 1965a) considers that crimp in wool fibres results from changes in follicle shape brought about by cyclic action of the arrector muscle attached to the follicle. That the follicle movement is the cause of crimp seems unlikely in the light of the above evidence, but may be an effect of the curved fibre moving through the follicle, or may be entirely independent of crimp formation.

"If the differential dyeing is an equilibrium phenomenon, there is clearly an excess of dyeing sites of both types [acidic and basic] in the orthocortex. Alternatively if the difference is in the rate of dye adsorption it could be accounted for by a contrast in the state of organization in the two segments [possibly accompanied by different cystine contents] without any major divergence in composition. Although the latter alternative is more likely, the settling of this question will probably have to await the analysis of the two segments obtained by a physical separation technique, with no possibility of chemical modification". [From Dusenbury and Menkart (1955)].

A method of separation is reported which does, I believe,

come up to the above criteria and from the analyses the latter view of Dusenbury and Menkart is favoured for the differential dyeing of the cortex.

2. SEPARATION AND ANALYSES OF CUTICLE FROM KERATIN FIBRES

[A] INTRODUCTION

Wool fibres can be dispersed into cuticle and cortical cells using ultrasonics [Bradbury and Chapman (1964)]. However, the ultrasonic treatment disperses mainly cortical cells, with only a small percentage of cuticle [Bradbury, Chapman and King (1965a)]. To obtain cuticle in a pure state, requires a lengthy and repetitive separation procedure [Bradbury and Chapman (1964)].

During an investigation of the material dissolved from wool shaken in 98% formic acid [Bradbury, Chapman and King (1965b)], it was observed that the formic acid solution became cloudy. For short shaking times [less than 12 hours], the sedimented material consisted of dispersed cuticle and skin

flakes, and for longer periods it also contained some cortical cells.

The amount of material dispersed was small, only 0.8% being shaken off in four hours, and 2% in ten hours [King (1967)]. The reason for the small dispersion is due to rapid felting, the fibres becoming felted into balls in less than 15 minutes.

In an attempt to increase the dispersion of cuticle, wool was stirred in formic acid with a mechanical stirrer. However, due to the wool felting around the stirrer blade immediately the stirrer was turned on, no material was dispersed.

The above results suggested that if the wool fibres could be moved rapidly through formic acid without felting, then not only would the amount of breakdown be increased, but possibly only cuticle would be dispersed. If this could be achieved the method would be superior to the "ultrasonic" method for obtaining cuticle.

It is shown that the rapid movement of wool through formic acid without felting can be achieved by the use of a Vibromix agitator, and as expected, mainly cuticle with a small percentage of cortical cells is dispersed into the formic acid.

Bradbury, Chapman and King (1965a, 1965b) have shown that the amino acid composition of cuticle [from wool fibres] differs considerably from the amino acid composition of wool, and suggested that due to the differences, the cuticle would be less crystalline than the cortex. This is in agreement with X-ray observations showing that the cuticle is amorphous

[Woods (1938), Lustig, Kondritzer and Moore (1945)]. Bradbury et al. also observed that the cuticle was less polar than the fibre as a whole.

After the development of a simple method to remove cuticle from wool using the Vibromix agitator, cuticle from other keratin fibres was obtained by the same method. Analyses show that the differences in the amino acid composition of the cuticles, when compared with the parent fibres, are similar to the differences observed between wool cuticle and wool.

Rogers (1964) has shown that the inner root sheath of hair follicles, and medullary cells of fibres, both contain the amino acid cutrulline. This is the first confirmed report of citrulline being incorporated in a protein.

During the amino acid analyses of the various keratin fibres and the cuticle from them, it was found that citrulline was present in all the hydrolysates. It is further shown that the citrulline present in the cuticle hydrolysates, comes from the cuticle, and not from medullary cell contamination of the cuticle samples, except in the case of Possum fur cuticle.

Rogers (1958) has also found that citrulline is partially converted to the amino acid ornithine during acid hydrolysis. In all the hydrolysates where citrulline was observed, ornithine was also found to be present.

[B] METHOD FOR PREPARATION OF CUTICLE AND POSSUM FUR MEDULLA

(i) Materials.

The following fibres were used in the investigations:

(a) Virgin Merino 64's wool fibres [unpigmented] from a pen fed sheep kept at the Division of Animal Physiology, C.S.I.R.O., Prospect, N.S.W. [Fleece No. SW291].

(b) Human hair fibres [pigmented, reddish-brown] from a 12 year old girl, supplied by Division of Animal Physiology, C.S.I.R.O.

(c) Unpigmented Alpaca fibres [Suri Blanco] supplied by the Division of Animal Physiology, C.S.I.R.O.

(d) Pigmented Alpaca fibres [brown] supplied by the Returned Soldiers and Sailors Woollen Mills, Geelong, Vic.

(e) A sample of Possum fur [pigmented, grey-brown] from the Returned Soldiers and Sailors Woollen Mills.

All the fibres, except wool, were medullated.

The method for cleaning the fibres, prior to removal of the cuticle, was as follows. The tips of the wool fibre staples were cut off and only the base portion used, since the tips through hydrolytic and oxidative weathering are considerably degraded [Louw (1960a)]. As the tips of the other fibres could not be easily recognized, they were not cut off. The grease on the fibres was removed with petroleum spirit [B.pt.60-80^{o}C] by Soxhlet extraction for 8 hours. After air drying, the fibres were then washed 6 times in distilled water at 50^{o}C, to remove

suint, dirt and some skin flakes.

The method of cleaning wool, used by Bradbury and Chapman (1964), was discontinued when it was found that the detergent [Gardinol BW] was absorbed by the fibres and could not be washed out [Bradbury et al. (1966)]. Although washing the fibres in water removes the dirt and suint, it does not remove as many skin flakes as washing in the detergent solution.

Reagents used throughout this investigation were A.R. grade unless otherwise stated. The formic acid was 98-100% A.R. grade, redistilled until residue free.

(ii) Preparation of Cuticle.

The method for removal of the cuticle from keratin fibres, using a Vibromix agitator, was initially developed as a quick and easy means of removing cuticle from wool fibres. Later, the method was applied to the removal of cuticle from other keratin fibres. Typical cuticle isolated from wool is shown in figure 1.4.

In the initial experiment, 2 gm of wool cut into short lengths [approximately 1-2 cm], was immersed in 50 ml formic acid and treated for 2 hours with the Vibromix agitator. The material dispersed was sedimented by centrifugation, the formic acid solution removed and the material washed with ethanol. Microscopic observation [using phase-contrast] showed that it consisted of cuticle with small amounts of cortical cells and skin flakes. In a second experiment, 1.0 gm of wool in 50 ml formic acid was agitated for one hour.

The dispersed material consisted of cuticle and a small amount of skin flakes - there were no cortical cells present.

To find the optimum operating conditions for removal of cuticle with the Vibromix agitator, varying concentrations of wool in formic acid were agitated for one hour periods. After filtering off the remaining fibres through a stainless steel wire mesh sieve, the dispersed material was sedimented by centrifugation. The formic acid solution was removed, freeze dried and weighed. The sedimented material was washed with ethanol, filtered through a tared No. 3 sintered glass crucible, washed with water, dried and weighed. The results are shown in table 2.1.

TABLE 2.1.

AMOUNT OF MATERIAL DISPERSED AND DISSOLVED FROM WOOL

Wool treated in 50 ml formic acid for 1 hour in the Vibromix agitator.

Weight of Air-dried Wool (gm)	Percentage Dispersed[a]	Percentage Dissolved[a]
0.50	0.6[b]	2.1[b]
0.75	0.6	2.6
1.00	0.9	2.6
1.50	2.7	2.3
2.00	2.1	3.0

[a] Average of duplicate experiments.

[b] Average of six experiments.

The percentage of material dispersed shows a similar trend to the results obtained for the dispersion of wool during ultrasonic disintegration treatments in formic acid [Bradbury and Chapman (1964)]. The main difference between the two methods is the concentration at which the maximum amount of material is dispersed, increasing from 1.0 gm per 50 ml formic acid for the ultrasonic treatment, to 1.5 gm per 50 ml when using the Vibromix agitator.

As stated earlier, the use of water instead of detergent for cleaning the fibres does not remove as many skin flakes from the fibres. As they are dispersed into the formic acid solution during the agitation treatment along with the cuticle, they have to be separated from the cuticle. This is difficult as they vary in size, ranging from the size of scale cells [approximately 4 micron square], up to about 50 to 100 micron square. However, they can be removed from the fibre before any cuticle is dispersed, by agitation of the fibres in 50 ml formic acid for 5 minutes. The fibres are filtered off, 50 ml fresh formic acid added and the agitation continued for the desired length of time to obtain cuticle.

For the removal of cuticle, this method offers several advantages over the ultrasonic method. These are as follows:

(a) In a 15 minute ultrasonic treatment 1.7% of wool is dispersed, but only a quarter of this material is cuticle - the remainder is mostly cortical cells with a small amount of skin flakes [Bradbury, Chapman and King (1965a)]. When using

the Vibromix agitator, the material dispersed is more than 90% cuticle, with some cortical cells and a few skin flakes, which makes purification of the cuticle easier. The small quantities of cortical cells present can be very easily removed by pouring an ethanolic suspension of the material through an 18 micron mesh sieve [Bradbury and Chapman (1964)].

(b) The vessel, containing the suspension of wool used in the ultrasonic disintegrator, has to be surrounded by a stirred ice/salt/water cooling bath to remove heat generated by cavitation in the liquid. The temperature is then only kept below 40°C with difficulty. When using the Vibromix agitator, however, there is no measureable temperature rise during the treatment. Since chemical reactions are in general hindered by lowering the temperature, there is less chance of any chemical degradation occurring during the latter treatment than the former.

(c) It is known that ultrasonic treatment modifies amino acids and soluble proteins in aqueous solution, and that water ultrasonically irradiated produces free radicals [reviewed in Bradbury, Chapman and King (1965a)]. By suitable experimentation, Bradbury et al. (1965a), showed that no significant changes in amino acid composition occurred during ultrasonic irradiation of wool. Although no experiments have been designed to see whether amino acids, soluble proteins, or wool fibres are modified by a treatment using the Vibromix agitator, this treatment appears to be much milder than the ultrasonic treatment.

No iodine is liberated from a solution of potassium iodide during agitation for 5 hours, implying that no free radicals are produced. Also there is less material dispersed and a smaller amount of material dissolved during treatments for the same time, as shown in table 2.2. Thus there is even less likelihood of any modification of the material using the Vibromix agitator.

TABLE 2.2.

AMOUNTS OF MATERIAL DISPERSED AND DISSOLVED DURING VARIOUS TREATMENTS

1 gm of wool in 50 ml formic acid treated for 1 hour

Treatment	Percentage Dispersed	Percentage Dissolved
Immersion	0	0.8
Shaking	0.2	1.1
Vibromix agitation	0.9	2.5
Ultrasonic treatment	21.6	4.0

(d) The Vibromix agitator is considerably less expensive, less complex, and much simpler to use than the ultrasonic disintegrator.

On substituting other keratin fibres for wool, and treating them in the Vibromix agitator, it was found that cuticle could also be removed from them. The conditions finally adopted for obtaining cuticle from all the fibres investigated are as follows:

1.5 gm of the fibre was cut into approximately 1-2 cm lengths, and immersed in 50 ml formic acid in a round bottomed glass cylinder 4 cm inside diameter by 10 cm long. The fibres were agitated using a Vibromix agitator, with the 3 cm diameter perforated glass plate placed in the centre of the suspension, and adjusted to operate at maximum amplitude. After the fibres were agitated for 5 minutes to disperse all the skin flakes, the formic acid was filtered off, the fibres teased apart and agitated for a further 55 minutes in 50 ml fresh formic acid. The resulting suspension [from the second agitation], was filtered through a stainless steel mesh sieve [35 mesh] to remove the remaining fibres, and then centrifuged. The formic acid was removed and the residue was washed six times with ethanol. The small quantity of cortical cells was removed from the cuticle by sieving the ethanolic suspension through an 18 micron mesh sieve [Bradbury and Chapman (1964)]. After checking the histological purity of the cuticle by phase-contrast microscopy, the ethanol was removed using a rotary evaporator and the residue finally dried at 105°C and weighed. The material dissolved in formic acid during the agitation was recovered by freeze drying. The results are shown in table 2.3.

The Possum fur was dispersed at a much faster rate than the other fibres. As it is thought that the dissolution of the cell membranes in formic acid weakens the structure of the fibre, and thus enables it to be dispersed by mechanical agitation [Bradbury, Chapman and King (1965b)], the rapid breakdown of

Possum fur possibly indicates that the membrane structure within this fibre is different to that of other keratin fibres. Either the membrane structure is more easily dissolved [in formic acid] than the cell membranes in the other keratin fibres, or in the other fibres there is some extra mode of attachment between adjacent cells besides the cell membranes, helping to hold the assemblage of cells together.

TABLE 2.3.

YIELD OF CUTICLE, CORTICAL CELLS AND DISSOLVED MATERIAL FROM FIBRES TREATED WITH THE VIBROMIX AGITATOR

1.5 gm fibres agitated in 50 ml formic acid for 55 mins.

Fibre	Cuticle [%]	Cortical Cells [%]	Dissolved Material [%]
Wool	1.0	0.1	2.5
Alpaca [white]	2.6	0.1	2.3
Alpaca [brown]	4.0	0.2	3.2
Human hair	0.8	0.1	2.8
Possum fur	7.0	2.8	5.3

The cuticle obtained from Possum fur, contained some material that differed slightly in appearance from cuticle. The amino acid analyses of this sample differed significantly from the amino acid composition of the other cuticles, and it was thought that this could be due to contamination of the cuticle with medullary cells. [See table 2.8 and discussion section 2C (ii)].

Prior to the analyses of Possum fur cuticle, it had been noticed that in the dispersed material there were some cortical cells with an unusual shape. Along one side of these cortical cells were a series of regularly spaced sharp protrusions, as can be seen in figure 2.1. These cortical cells have been termed serrated cortical cells [compare with normal cortical cells shown in figure 1.5]. At the time no explanation could be given for the protrusions, but when the amino acid analyses suggested that there could be medulla contaminating the cuticle sample, the explanation of them became clear. The medulla of Possum fur, as can be seen in figure 2.2, consists of regular oval shaped cells, and it is obvious that the protrusions on the serrated cortical cells fit in between adjacent medullary cells. It has also been observed that Kangaroo fur contains serrated cortical cells [O'Shea (1967)]. Thus it appears that all medullated fibres will contain serrated cortical cells.

In the follicle, before keratinization of the actively growing fibre [see section 1A], the cortical cells are soft and pliable. As the cells get pushed up from the bulb into the neck of the follicle, the cortical cells must get moulded around the medulla cells, which are "pressure resistant" and not pliable [Auber (1952)]. During the keratinization process, the cells become hardened due to the formation of disulphide bonds and dehydration, and the protrusions become a permanent feature of the cortical cells which lie adjacent to medullary cells.

The serrated cortical cells can be used to check the

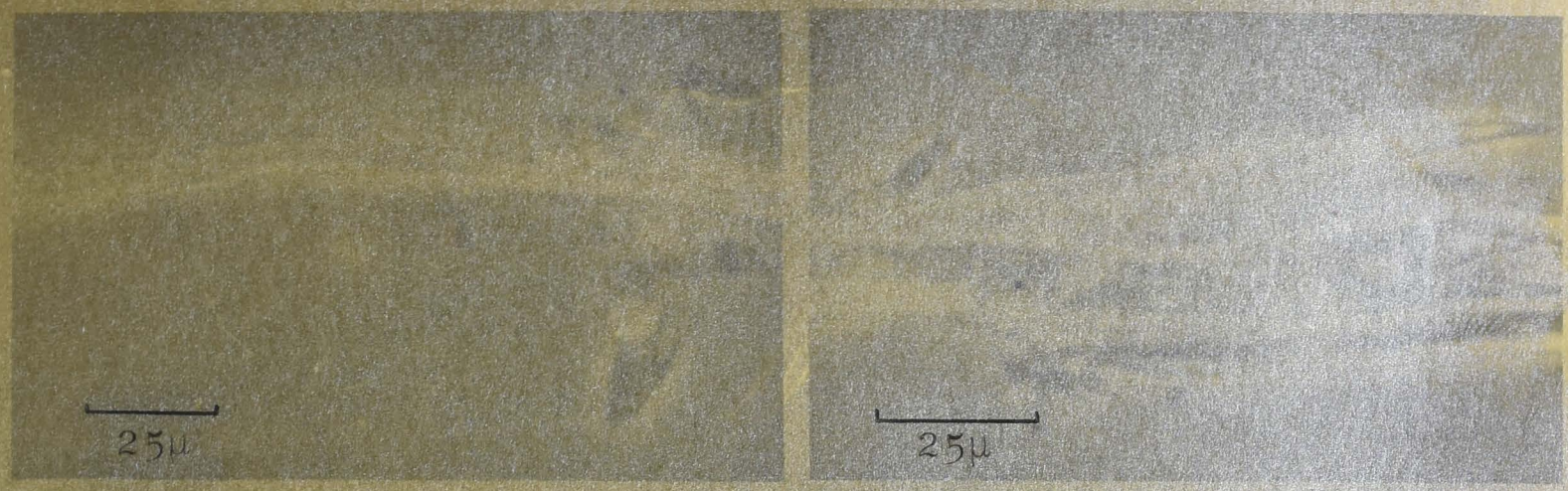


Figure 2.1. Phase-contrast light micrograph of cortical cells isolated from Possum fur.

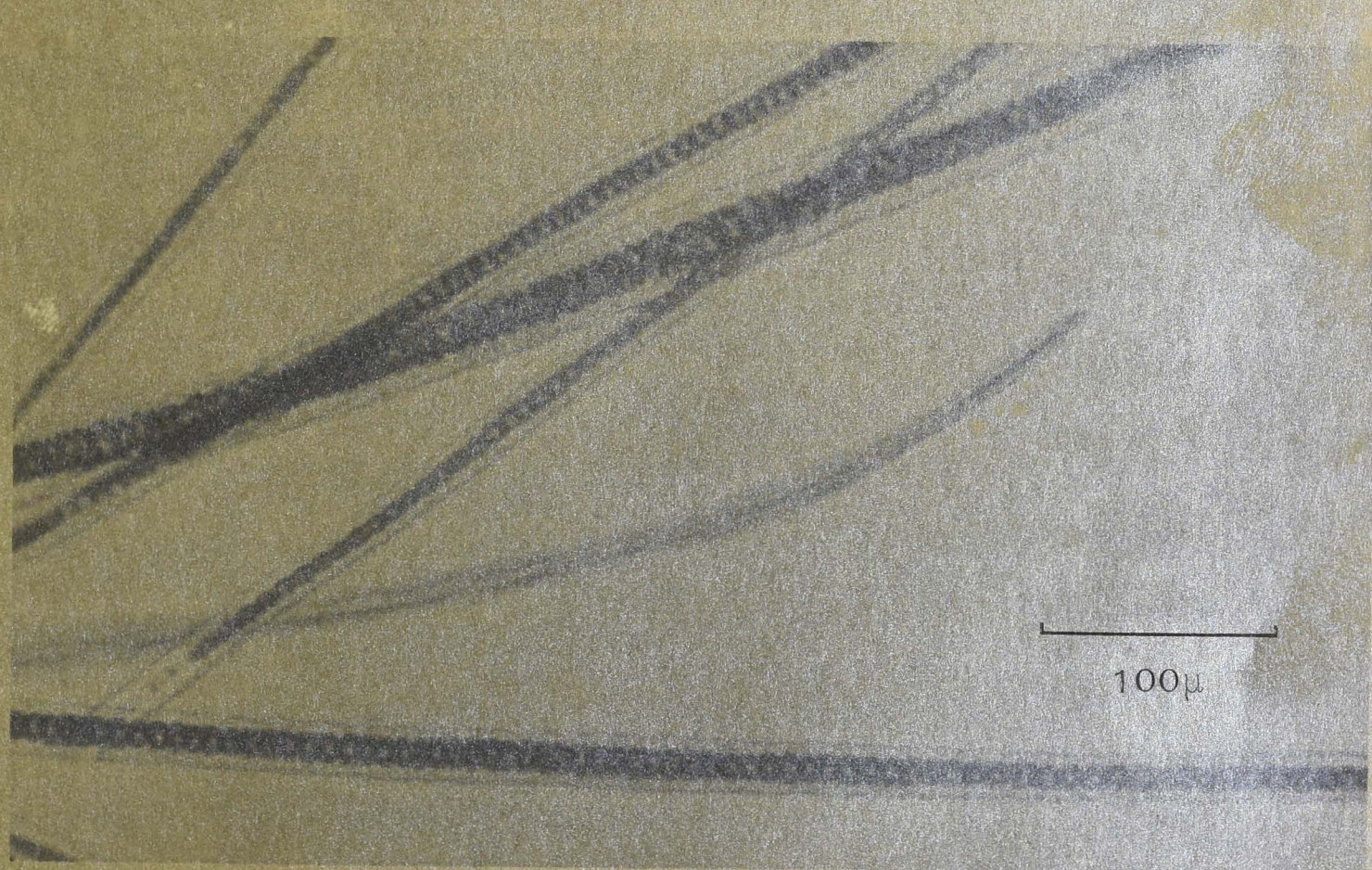


Figure 2.2. Light micrograph of Possum fur showing the medulla

purity of the cuticle preparations. After the Vibromix treatment, if there are serrated cortical cells present in the dispersed material, then one can assume that medullary cells have also been dispersed. Of the material dispersed from the various fibres, only the material from the Possum fur contained serrated cortical cells, and thus medullary cells. This also implies that all the other cuticle samples were free of medullary cells [assuming that all medullated fibres contain serrated cortical cells].

Using the above criteria, a milder treatment was sought for the removal of cuticle from Possum fur. Reduction in the time of the Vibromix agitation treatment to 5 minutes, and even shaking of the Possum fur in formic acid for 5 minutes by hand, still produced a dispersion of cuticle containing some serrated cortical cells and thus some medullary cells. No method was found to either produce cuticle free of medullary cells, or to purify the cuticle sample adequately.

(iii) Preparation of Possum Fur Medulla.

The medulla was prepared by the method outlined by Matoltsy (1953), which is as follows:

10 gm of Possum fur was stirred in 400 ml of 0.7N potassium hydroxide for 1 hour, which partially dissolves the cuticle and cortex and suspends in the solution dust particles and impurities from the fibres. The alkali was removed by repeated suspension of the fibres in distilled water. The clean material was then stirred for 1 hour in 200 ml 3N potassium

hydroxide. After this, the suspension was allowed to stand at room temperature for 17 hours, during which time the cortex completely dissolved while the medullary cells sedimented to the bottom of the beaker. After discarding the supernatant, the suspension was filtered three times through coarse glass wool to remove the undissolved particles of the fur. The medullary cells were separated from a small amount of finely divided cell fragments by centrifuging for 30 minutes at 1400 G. The sediment of medullary cells was resuspended successively in 1.5N potassium hydroxide, 0.7N potassium hydroxide and finally three times in distilled water. Microscopic examination of this material showed that it consisted entirely of medullary cells, cuboidal in shape. They resembled the unidentified material in the Possum cuticle preparations. The material was rendered alkali free by repeated washings in distilled water.

[C] AMINO ACID ANALYSES

(i) Experimental.

Initially, twenty-one hour amino acid analyses were performed as follows:

The material for analysis was dried in vacuo at 100°C for 1 hour and approximately 4 mgm was weighed accurately. One ml of 6N hydrochloric acid [constant boiling point acid produced by distilling 50% A.R. hydrochloric acid] was added to the sample in a glass tube, sealed under vacuum and hydrolysed for 24 hours at 110°C. The solution was evaporated nearly to dryness using a rotary evaporator, and the hydrolysate diluted to 10.0 ml with 0.1N hydrochloric acid. One ml of the solution was loaded onto an ion-exchange column [0.63 cm diameter by 125 cm length containing an 8% cross-linked sulphonated polystyrene resin] of a Technicon amino acid analyser [Piez and Morris (1960)]. Known amounts of the amino acids taurine, norleucine and α-amino-β-guanidinopropionic acid were also run routinely as internal standards to correct for small variations in behaviour of the amino-acid analyser. The effluent from the column was mixed with ninhydrin, heated at 95°C for 15 minutes, and the colour produced measured by two colorimeters, one set at 440 millimicrons and the other at 570 millimicrons.

Later, the amino acid analyser was modified by the addition of a second ion-exchange column, the same as above. The time

to complete an analysis was decreased to 10 hours by increasing the flow rate of the eluting buffer, so that two complete analyses could be performed in 24 hours. The above hydrolysis procedure had to be modified as follows: Approximately 3 mgm of material was dried at 100°C in vacuo for one hour and hydrolysed in 1 ml 6N hydrochloric acid [constant boiling point acid] under vacuum in a sealed glass tube for 24 hours at 110°C. The solution was evaporated nearly to dryness and diluted to 5.0 ml with a solution of 12.5% sucrose in 0.1N hydrochloric acid. 0.5 ml of the hydrolysate solution was then injected on to the top of each ion-exchange column by means of a sample injection device. This was followed by 0.2 ml of the internal standard solution. By means of a programmer, the amino acids were eluted from one column and then from the other column. The effluent from each column in turn, passed through the same analyser system as outlined above.

A sample of a Beckmann standard amino acid mixture was run regularly [once every 10 - 12 runs] to calibrate the analyser, so that accurate quantitative analyses could be performed. All hydrolysates were analysed at least twice and the results averaged.

The amino acid citrulline was present in some of the hydrolysates [see discussion 2 C (iii)]. Under the normal operating conditions of the amino acid analyser, citrulline and proline emerge from the ion-exchange column together and are recorded on the chromatogram as a single peak. However, a

quantitative result can be obtained for both amino acids.

The maximum absorbance of the colour produced by the reaction of most amino acids [including citrulline] with ninhydrin, is at about 570 millimicrons. For a few amino acids, e.g. the hydroxyprolines and proline, the maximum absorbance of the colour is at 440 millimicrons. For proline, the ratio of the area under the 440 millimicron trace (A_{440}) to the area under the 570 millimicron trace (A_{570}) is 6.0,

$$\text{i.e. for proline } \frac{A_{440}}{A_{570}} = 6.0.$$

$$\text{If } \frac{A_{440}}{A_{570}} < 6.0$$

then citrulline is present in the hydrolysate. Further, from the nett height of the two peaks, the amounts of both citrulline and proline can be calculated [Holy (1966)].

Typical traces in the region of glutamic acid and proline, when citrulline is absent (a) and present (b) in a hydrolysate, are shown in figure 2.3.

To obtain accurate values for both citrulline and proline, it is desirable to resolve them on the ion-exchange column. This can be accomplished by alteration of the temperature of the ion-exchange resin.

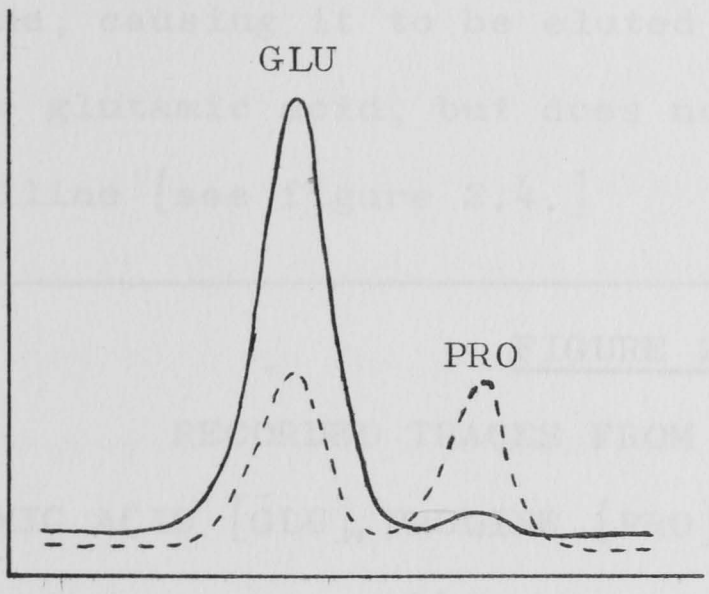
When the temperature of the ion-exchange column was dropped from 60°C [the normal operating temperature] to 40°C, then proline and citrulline were separated but proline emerged

FIGURE 2.3.

RECORDED TRACES FROM A CHROMATOGRAM IN THE REGION OF GLUTAMIC ACID [GLU] AND PROLINE [PRO].

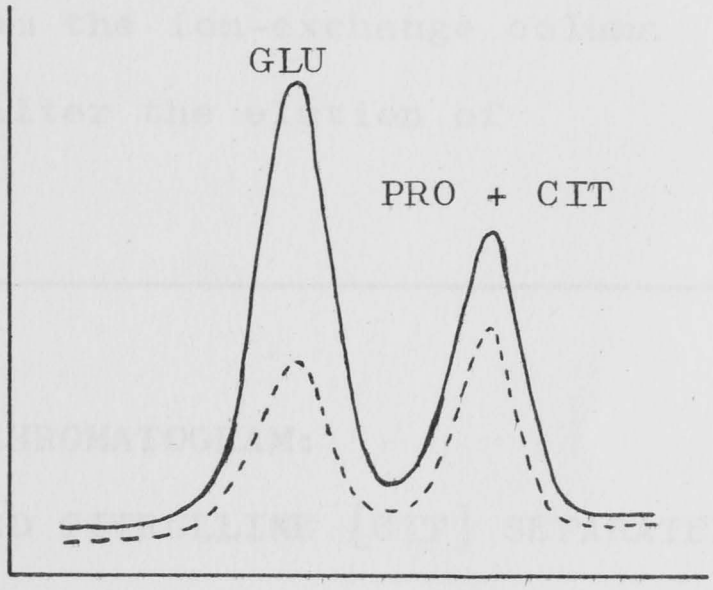
--- 440 millimicron trace

—— 570 millimicron trace



(a)

Traces from hydrolysates which contain no citrulline.



(b)

Traces from a hydrolysate which contains a large amount of citrulline [CIT]

from the column coinciding with glutamic acid. Also, the resolution of aspartic acid, serine and threonine was impaired. After several more experiments, conditions were found which did resolve citrulline and proline without impairing resolution of any other amino acids, and is as follows, when using the analyser set for 21 hour analyses: If citrulline is present in the first analysis of a hydrolysate as indicated by

$$\frac{A_{440}}{A_{570}} < 6.0$$

then for the duplicate analysis, the temperature of the ion-exchange column is lowered from 60°C to 15°C when aspartic acid is just emerging from the ion-exchange column.* The temperature is returned to 60°C after the proline peak has been recorded. This procedure changes the relative elution of proline, causing it to be eluted from the ion-exchange column before glutamic acid, but does not alter the elution of citrulline [see figure 2.4.]

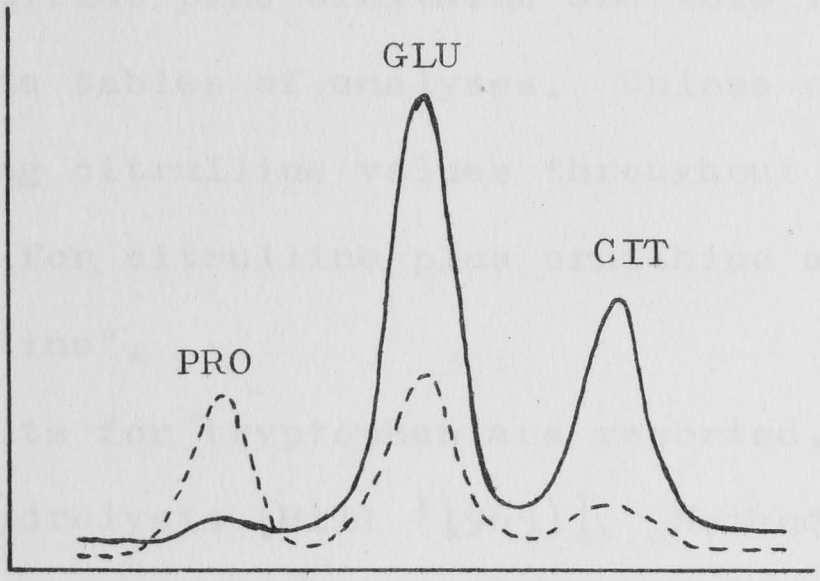
FIGURE 2.4.

RECORDED TRACES FROM A CHROMATOGRAM:

GLUTAMIC ACID [GLU], PROLINE [PRO] AND CITRULLINE [CIT] SEPARATED

--- 440 millimicron trace

—— 570 millimicron trace



* For the amino acid analyser used, the temperature was altered to 15°C, 20 minutes before aspartic acid was recorded on the chart. [There is a delay of 20 mins. from the time of emergence from the column to being recorded on the chart, due to passing through the analyser]. This time was determined from the first analysis.

(ii) Results.

All the results are expressed as mole per 100 moles of the amino acids in the hydrolysate, and for convenience this is abbreviated to mole per cent. Included with every analysis is the percentage recovery of anhydroamino acids [RAAA%]. It should be noted that when results are expressed in mole per cent, direct comparison can be made between analyses, without having to allow for the percentage of protein in the sample [i.e. the percentage recovery of anhydroamino acids]. This is not the case if the results are expressed in other units, except as N % total N in the sample.

Rogers (1958) has observed that citrulline is partially converted to ornithine during acid hydrolysis. Thus both citrulline and ornithine values are reported in the amino acid analyses. For comparison however, one should use the total of citrulline plus ornithine and this value is also included in the tables of analyses. Unless otherwise stated, when discussing citrulline values throughout this thesis, the value will be for citrulline plus ornithine and will be termed "total citrulline".

No results for tryptophan are reported, as it is destroyed during acid hydrolysis [Hill (1965)]. Methods for analysing tryptophan are outlined by Crewther et al. (1965); none have been applied to any of the samples.

Separate analyses have not been made for glutamine or asparagine. As these amino acids are converted quantitatively to glutamic acid and aspartic acid during acid hydrolysis

[Hill (1965)], the glutamic acid and aspartic acid values reported are the sum of the values for the acid and the acid amide in each case.

To correct for destruction of some amino acids [serine. threonine and possibly cystine, aspartic acid, glutamic acid, lysine, arginine, tyrosine and proline] and incomplete liberation of others [valine, isoleucine and leucine] during hydrolysis, analyses of hydrolyses carried out for various times should be performed. The results after plotting, are extrapolated to zero time to give accurate values for each amino acid [Hill (1965)].

The corrections that have to be applied to the amino acid values from wool hydrolysates are small, the maximum corrections being for serine [7.5%], threonine [7.0%], tyrosine [5.5%] and cystine [3.0%] [Bradbury, Chapman and King (1965a)]. These authors also found that all the amino acids were liberated during a 24 hour hydrolysis. As no hydrolyses for extended periods have been carried out, the analyses reported are uncorrected for hydrolytic losses.

Amino acid analyses of Merino 64's wool [Bradbury, Chapman and King (1965a)] and cuticle dispersed from this wool by shaking in formic acid [Bradbury, Chapman and King (1965b)], are shown in table 2.4.

In table 2.5, the amino acid composition of the white Alpaca [Suri Blanco] fibre and cuticle samples is shown, and in table 2.6 that of the brown Alpaca fibre and cuticle samples.

TABLE 2.4.

AMINO ACID ANALYSES [MOLE %] OF MERINO WOOL[a] AND CUTICLE[b]

Amino Acid	Merino Wool[a]	Merino Wool Cuticle[b]
Cysteic acid	0.08	0.23
Aspartic acid	6.36	3.15
Threonine	6.50	4.69
Serine	10.26	13.34
Glutamic acid	11.93	8.28
Proline	5.94	10.12
Citrulline	0.045[c]	0.32[c]
Glycine	8.61	9.84
Alanine	5.33	5.61
Valine	5.53	6.94
Half cystine	10.43	15.89
Methionine	0.50	0.36
Isoleucine	3.13	2.49
Leucine	7.70	5.53
Tyrosine	3.96	2.98
Phenylalanine	2.92	1.63
Ornithine	0.028[c]	0.16[c]
Lysine	3.06	2.82
Histidine	0.93	1.00
Arginine	6.83	4.63
Total citrulline	0.073[c]	0.48[c]
RAAA [%][d]	95.5	99.2

[a] From Bradbury, Chapman and King (1965a).

[b] From Bradbury, Chapman and King (1965b).

[c] From King (1967)

[d] Recovery of anhydroamino acids.

TABLE 2.5.

AMINO ACID ANALYSES [MOLE %] OF WHITE ALPACA [SURI BLANCO] FIBRES AND CUTICLE

Amino Acid	Alpaca [White]	Alpaca [White] Cuticle
Cysteic acid	0.43	1.57
Aspartic acid	6.32	3.21
Threonine	6.23	4.58
Serine	10.72	12.26
Glutamic acid	13.50	8.55
Proline	7.21	12.79
Citrulline	0.43	0.94
Glycine	7.15	8.82
Alanine	5.42	5.76
Valine	5.60	7.36
Half cystine	11.66	17.92
Methionine	0.41	0.21
Isoleucine	2.78	1.58
Leucine	7.12	4.79
Tyrosine	2.08	0.78
Phenylalanine	2.41	1.10
Ornithine	0	0.38
Lysine	2.67	1.99
Histidine	0.78	0.67
Arginine	7.07	4.68
Total citrulline	0.43	1.32
RAAA [%][a]	91.2	77.2

[a] Recovery of anhydroamino acids.

TABLE 2.6.

AMINO ACID ANALYSES [MOLE %] OF BROWN ALPACA FIBRES AND CUTICLE

Amino Acid	Alpaca [Brown]	Alpaca [Brown] Cuticle
Cysteic acid	0.52	2.31
Aspartic acid	6.62	4.07
Threonine	6.17	5.00
Serine	10.56	12.45
Glutamic acid	13.37	9.16
Proline	6.96	11.35
Citrulline	0.30	0.68
Glycine	7.75	9.04
Alanine	5.54	6.08
Valine	5.43	8.11
Half cystine	10.78	12.91
Methionine	0.44	0.29
Isoleucine	2.97	1.99
Leucine	7.38	5.62
Tyrosine	2.36	1.44
Phenylalanine	2.84	1.37
Ornithine	0	0.47
Lysine	2.46	2.26
Histidine	0.76	0.62
Arginine	6.78	4.77
Total citrulline	0.30	1.15
RAAA [%][a]	80.7	66.8

[a] Recovery of anhydroamino acids.

TABLE 2.7.

AMINO ACID ANALYSES [MOLE %] OF POSSUM FUR "CUTICLE"

Amino Acid	Sample 1	Sample 2	Average
Cysteic acid	0.25	0.22	0.24
Aspartic acid	3.54	3.65	3.60
Threonine	3.87	3.78	3.82
Serine	11.20	10.44	10.82
Glutamic acid	15.52	15.39	15.46
Proline	6.85	6.96	6.91
Citrulline	3.20	3.11	3.15
Glycine	10.76	10.79	10.78
Alanine	4.67	4.80	4.73
Valine	4.84	5.10	4.98
Half cystine	15.43	14.84	15.14
Methionine	0.46	0.40	0.43
Isoleucine	1.78	1.86	1.82
Leucine	5.38	5.64	5.52
Tyrosine	2.09	2.20	2.14
Phenylalanine	1.52	1.53	1.52
Ornithine	1.34	2.08	1.71
Lysine	4.19	4.07	4.13
Histidine	0.52	0.52	0.52
Arginine	2.60	2.62	2.61
Total citrulline	4.54	5.19	4.86
RAAA [%][a]	74.2	75.0	74.6

[a] Recovery of anhydroamino acids.

TABLE 2.8.

AMINO ACID ANALYSES [MOLE %] OF POSSUM FUR AND "CUTICLE"

Amino Acid	Possum Fur	Possum Fur "Cuticle"[a]
Cysteic acid	0.23	0.24
Aspartic acid	6.31	3.60
Threonine	5.03	3.82
Serine	8.33	10.82
Glutamic acid	13.10	15.46
Proline	7.10	6.91
Citrulline	0.09	3.15
Glycine	9.35	10.78
Alanine	6.12	4.73
Valine	5.93	4.98
Half cystine	11.65	15.14
Methionine	0.72	0.43
Isoleucine	3.22	1.82
Leucine	7.18	5.52
Tyrosine	3.20	2.14
Phenylalanine	1.89	1.52
Ornithine	0.47	1.71
Lysine	3.09	4.13
Histidine	0.94	0.52
Arginine	6.05	2.61
Total citrulline	0.56	4.86
RAAA [%] [b]	88.3	74.6

[a] From table 2.7, column 3.

[b] Recovery of anhydroamino acids.

TABLE 2.9.

AMINO ACID ANALYSES [MOLE %] OF HUMAN HAIR AND CUTICLE

Amino Acid	Human Hair	Human Hair Cuticle
Cysteic acid	0.27	0.93
Aspartic acid	5.29	3.26
Threonine	7.44	3.90
Serine	11.90	11.48
Glutamic acid	12.49	10.00
Proline	8.23	9.45
Citrulline	0.12	0.09
Glycine	5.76	10.51
Alanine	4.52	6.06
Valine	5.38	8.41
Half cystine	16.30	19.00
Methionine	0.51	0.41
Isoleucine	2.58	2.13
Leucine	5.93	4.72
Tyrosine	2.05	1.41
Phenylalanine	1.66	1.21
Ornithine	0	0.61
Lysine	2.42	3.43
Histidine	0.83	0.45
Arginine	6.33	2.52
Total citrulline	0.12	0.70
RAAA [%][a]	92.6	75.7

[a] Recovery of anhydroamino acids.

TABLE 2.10.

AMINO ACID ANALYSES [MOLE %] OF HUMAN HAIR.

Amino Acid	Present Work[a]	Simmonds (1958)	Lang and Lucas (1952)
Cysteic acid	0.27	-	-
Aspartic acid	5.29	5.27	3.59
Threonine	7.44	6.88	8.67
Serine	11.90	10.81	12.57
Glutamic acid	12.49	11.24	10.86
Proline	8.23	9.56	4.57
Citrulline	0.12	-	-
Glycine	5.76	6.50	7.41
Alanine	4.52	4.38	4.07
Valine	5.38	6.22	5.73
Half cystine	16.30	18.06	18.17
Methionine	0.51	-	0.55
Isoleucine	2.58	2.69	4.38
Leucine	5.93	5.89	7.48
Tyrosine	2.05	1.60	1.39
Phenylalanine	1.66	1.81	1.68
Ornithine	0	-	-
Lysine	2.42	2.27	1.53
Histidine	0.83	0.79	0.90
Arginine	6.33	6.04	5.88
Tryptophan	-	-	0.56
RAAA [%][b]	92.6	83.7	86.0

[a] From table 2.9, column 1

[b] Recovery of anhydroamino acids.

Duplicate analyses are shown in table 2.7 for Possum fur "cuticle" samples, from one hour Vibromix agitations. After analysing the "cuticle" sample and finding that the recovery of anhydroamino acids was only 74.2%, a second sample of "cuticle" was prepared and analysed. The recovery, 75.0%, and the value for each amino acid was the same, within experimental error, as found in the first analysis. These results show that the method of preparation of cuticle is reproducible, as well as the technique involved in hydrolysing the protein samples.

In table 2.8, the analysis of Possum fur and the average analysis of Possum fur cuticle [from table 2.7] is shown. Human hair and Human hair cuticle amino acid compositions are tabulated in table 2.9.

Table 2.10 shows the amino acid analyses of Human hair performed by Lang and Lucas (1952), and Simmonds (1958), which are the only complete analyses for Human hair reported in the literature. Also shown for comparison is the amino acid analysis of Human hair from table 2.9. The agreement between the analysis obtained in this present work and the analysis of Simmonds is very good. All the results are within experimental error, except for the amino acids proline, glycine, valine and tyrosine. The results of Lang and Lucas, obtained by a microbiological method, differ from the others considerably.

Microanalysis of the fibres and cuticle preparations are shown in table 2.11. All the cuticle samples have a considerably

higher ash content than the parent fibre and it appears that all the mineral material is concentrated in the cuticle. This is the case with wool, where there is known to be 10% cuticle [Bradbury and King (1967)]. This is not surprising if the material [giving rise to the ash] has been picked up by the fibre after emergence from the follicle and not incorporated in the cells [which will form the fibre] in the bulb of the follicle.

TABLE 2.11

MICROANALYSES OF FIBRES AND CUTICLES

All results expressed as per cent on dry weight.

Material	C	H	N	Ash
Wool	48.75	6.90	15.28	0.22
Cuticle	-	-	-	2.27
Alpaca (white)	47.38	6.89	16.21	0.61
Cuticle	-	-	-	1.86
Alpaca (brown)	48.49	6.76	16.25	0.34
Cuticle	42.61	6.12	13.86	6.65
Human hair	46.6	7.1	15.15	0.40
Cuticle	-	-	-	4.9
Possum fur	46.88	6.93	14.75	0.35
Cuticle	44.13	6.55	12.75	6.08

It is interesting that in the two cuticle samples that have been fully analysed, the nitrogen contents are low, which indicates that the protein content will be low. This is actually observed [see table 2.6 and 2.7].

(iii) Discussion.

The recovery of anhydroamino acids from hydrolysates of the fibres, varies from 80.7% to 95.5%. The latter figure for wool is acceptable, but the figures for the other fibres of 92.6 for Human hair, 91.2% for Alpaca [white] fibres, 88.3% for Possum fur and 80.7% for the pigmented Alpaca [brown] fibres are very low.

There are several possible reasons for a low recovery of amino acids and these are:

(a) losses during the hydrolytic procedure

(b) the presence of non-protein material such as melanin granules [pigment], lipids, mineral material [ash], nucleic acids and carbohydrates.

In order to allow for the above non-protein contaminants, analyses have been performed or obtained for some of the items and for the others, estimates have been made. The results are reported in table 2.12 [which should be read in conjunction with the following text].

The recovery of anhydroamino acids from analyses of three wool samples [for Dr. Lang, Gordon Institute of Technology, Geelong, Victoria, but not reported here] were 97.1%, 98.6% and 95.7%. From these results it can be seen that the hydrolytic procedure is reproducible and apart from tryptophan, which is destroyed during acid hydrolysis, no appreciable losses occur during the procedure.

TABLE 2.12

CONSTITUENTS OF THE FIBRES

All values expressed as per cent of dry weight

Fibre	Protein		Tryptophan	Pigment	Lipid + Nucleic acid	Ash[c]	Total	
	RAAA[a]	Calculated[b]					g	h
Wool	95.5	94.5	0.9[d]	0	0.7	0.3	97.4	96.4
Human hair	92.6	94.5	0.9[e]	2[f]	0.7[f]	0.4	96.6	98.5
Alpaca (white)	91.2	98.5	0.8[f]	0	0.7[f]	0.6	93.3	100.6
Alpaca (brown)	80.7	100.3	0.8[f]	2.2	0.7[f]	0.3	84.7	104.3
Possum fur	88.3	91.4	0.8[f]	3.1	0.7[f]	0.4	93.3	96.4

[a] Recovery of anhydroamino acids: From table 2.4, 2.5, 2.6, 2.8 and 2.9.

[b] See text.

[c] From table 2.11.

[d] From Graham and Statham (1960): See text.

[e] From Lang and Lucas (1952): See table 2.10.

[f] Value is an estimate: See text.

[g] Total of values in columns 2[a], 3, 4, 5, 6.

[h] Total of values in columns 2[b], 3, 4, 5, 6.

Simmonds (1954) reports that wool contains 1.9% [w/w] tryptophan. Later (1955), from analyses of three wools, he concludes that the tryptophan content is 2.3%. However he states that his method of analysis [for tryptophan] is suspect and that the values are too high. Graham and Statham (1960) estimated the tryptophan content of 31 samples of wool and the values obtained [for the root end of the fibres] varied from 0.88% to 1.07%, with a mean of 0.95%. Thus Simmonds' (1954, 1955) results are too high and the value for all types of wool is approximately 0.95% [w/w].

Lang and Lucas (1952) found that Human hair contains 0.93% [w/w] tryptophan [see table 2.10]. Graham and Statham (1960) report a value of 0.54% for Human hair. For several other keratin fibres they report values ranging from 0.82% to 0.97%. Thus it appears that all keratin fibres have substantially the same tryptophan content of about 0.8% - 0.9% [w/w]. A value of 0.8% [w/w] has been used for Possum fur and the two Alpaca fibre samples in table 2.12.

Carbohydrates are not present in the fibres, as both the anthrone test [Dreywood (1946)] and the Molisch test [Vogel (1956)] give negative results when applied to the fibres.

The colour of fibres [and skin, eye tissue etc.] is due to the presence of the pigment melanin in the material. The melanin is in the form of granules which range in size from spheres of 0.05 to 0.6 microns in diameter, to rods varying in size from 0.25 x 0.4 microns to 0.43 x 1.33 microns [Laxer et al.

(1954)]. Associated with the melanin granules and probably non-ionically bound, is a small percentage of iron [about 0.35% in wool melanin, which is equivalent to about 0.02% of the weight of the fibre] [Laxer and Whewell (1954)], and also copper and zinc [Stein (1955)].

Methods for estimating the amount of melanin present in fibres depend on the chemical stability of the granules. Most workers have used the method of Einsele (1937), in which the keratin is dissolved by refluxing the fibres in 6N hydrochloric acid for 1-1/2 hours. However, Laxer et al. (1954) state that melanin isolated by this method is considerably modified, and consider that isolation of melanin by refluxing the fibre in a solution of phenol-hydrate containing thioglycollic acid [PHT solution] is superior. Green and Happey (1965), after an infra-red examination of melanin granules, conclude that isolation of them by 6N HCl probably causes no less damage than isolation using PHT solution. Birbeck and Barnicot (1959) also found that isolation by heating the fibres in 3N HCl causes little or no damage.

The melanin content of the Possum fur and the Alpaca [brown] fibres was thus determined using the method outlined by Green and Happey (1965). This consisted of refluxing approximately 0.5 gm [known dry weight] of the fibre with 20 ml 6N HCl, for 4 hours. The undissolved melanin granules were filtered through a tared No. 3 sintered glass crucible, washed with water, acetone and ether, dried at 110^{o}C and weighed.

As there was only a small quantity of Human hair remaining after preparing the cuticle, no melanin content was performed on this fibre. Einselle (1937) found that Human hair contained from 0.7% to 7.4% melanin. As the hair analysed was a very light red-brown in colour, a value of 2% has been estimated for its melanin content for table 2.12.

Whether the small amount of metal ions associated with melanin [see above] will cause any breakdown or loss of amino acids during hydrolysis, is unknown. It is known that tyrosine is destroyed in the presence of Fe^{++} or Cu^{++} ions [Homer (1915)] and cystine by Cu^{++} ions [Olcott and Fraenkel-Conrat (1947)]. However, Kimmel, Markowitz and Brown (1959) report that there were no losses during hydrolysis of erythrocuprein [from human erythrocytes], although the protein contains 0.38% copper. There does not appear to be any comprehensive study of the action of metals, during hydrolysis of proteins, reported in the literature. Therefore no estimate can be made for the losses during hydrolysis of the pigmented fibres, if any occur at all.

The amount of other non-protein material, lipid, nucleic acids and ash, is approximately 1% in wool [Alexander, Hudson and Earland (1963)]. There is about 0.3% ash in wool [see table 2.11]. King (1967) has found that the maximum amount of "lipid" in wool is about 0.73% [obtained from immersion of wool in formic acid for various times]. This "lipid" material also probably contains the nucleic acids [if any are present] from the nuclear remnants. As the structure of all the keratin fibres

is similar, it is assumed that there would also be less than 1% of lipid and nucleic acid material in them. Thus a value of 0.7% has been assumed for all the fibres for "lipid + nucleic acid" content, in table 2.12. The values for the ash contents are from table 2.11.

The total of the constituents ['g', table 2.12], indicates that [within experimental error] all the Human hair and wool has been accounted for. The 7% deficit for the white Alpaca and Possum fur, and 15% deficit in the brown Alpaca sample, is apparently due to loss of some of the protein during hydrolysis.

As a check on the percentage protein recorded in table 2.12, the total nitrogen contribution from each amino acid was calculated and expressed as a percentage of the total nitrogen in the fibre. The values, together with those for the Alpaca [brown] cuticle and Possum fur "cuticle", are shown in table 2.13. These values give the true recovery of protein in the sample [if all the nitrogen is only derived from the protein].

The results in table 2.13 indicate that in the wool, Human hair and Possum fur analyses, all of the protein nitrogen is accounted for [within experimental error]. In all the other samples, the percentage protein accounted for is low, assuming that all the nitrogen is derived from the protein. There is the possibility of unidentified nitrogen compounds present in the samples, which do not react with ninhydrin, or are not eluted from the ion-exchange column of the amino acid analyser. Failing this, the low recoveries can only be due to

hydrolytic losses, or destruction of some or all of the amino acids in the samples during hydrolysis.

TABLE 2.13

RECOVERIES OF NITROGEN FROM FIBRE ANALYSES AND TWO CUTICLE ANALYSES.

Sample	Total N in Hydrolysate[a] [gm/100 gm sample]	Total N in Sample[b] [gm/100 gm sample]	Percentage Recovery of N
Wool	15.43	15.28	101.1
Human hair	14.86	15.15	98.0
Alpaca [white]	14.86	16.21	91.6
Alpaca [brown]	13.04	16.25	80.4
Cuticle	10.78	13.86	77.7
Possum fur	14.25	14.75	96.6
Cuticle	11.91	12.75	93.5

[a] Calculated from amino analyses.

[b] From table 2.11

If an allowance is made for the low recovery of protein [as indicated in table 2.13] and the "true" protein content of the sample is calculated [shown in table 2.12 column 2 under "calculated"], then all of the material in all of the fibres is accounted for [see second column (h) under "Total", table 2.12]. Also, this indicates that the assumptions made for the unknown values in table 2.12, were of the correct order of magnitude.

Reports of low recoveries for keratin fibres can be found in the literature. For Human hair, Simmonds (1958), obtained a value of 83.7% and Lang and Lucas (1952) 86.0% [see table 2.10]. Ward, Binkley and Snell (1955) report a value of 73.3% for Mohair, and Rogers (1958) 72.8% for Rabbit fur. King (1967), however, obtained a 93.1% recovery for Rabbit fur and 99.5% for Mohair.

The analyses of the two Possum fur "cuticle" samples and the recovery of 74.2% and 75.0% of anhydroamino acids respectively, again indicates that the hydrolytic procedure is reproducible and, as stated above, there appears to be no hydrolytic losses apart from tryptophan. The tryptophan content of wool cuticle has been reported as 0.6% [Inglis, Leaver and Lennox (1965)] and that of Human hair cuticle as 0.2% [Lustig, Kondritzer and Moore (1945)]. Thus this has little effect on the low recoveries.

The tests for carbohydrate [Molisch test and anthrone test] gave negative results on all cuticle samples. Also, there will be no lipid or nucleic acid material in the cuticle samples, as this would be dissolved by the formic acid during the preparation treatment.

In pigmented wool fibres, the melanin is distributed throughout the cortex and cuticle, whereas in Human hair the cuticle is unpigmented [Auber (1952)]. The cuticle of the other pigmented fibres [brown Alpaca and Possum fur] have not been investigated, but could contain some melanin. Unfortunately, the amount of cuticle obtained from

the fibres was too small to allow analyses for melanin to be performed. Ash analyses have been obtained and are tabulated in table 2.11.

In table 2.14, the data on constituents of the cuticle are collected. It can be seen that the only cuticle sample for which all the material can be accounted for, is the wool cuticle sample.

TABLE 2.14.

CONSTITUENTS OF THE CUTICLE SAMPLES

All values expressed as per cent of dry weight.

Cuticle from	Protein[a]	Ash[b]	Total
Wool	99.2	2.3	101.5
Human hair	75.7	4.9	80.6
Alpaca [white]	77.2	1.9	79.1
Alpaca [brown]	66.8	6.7	73.5
Possum fur	74.6	6.1	80,7

[a] Recovery of anhydroamino acids: From tables 2.4 to 2.9.

[b] From table 2.11.

There was enough Alpaca [brown] cuticle sample and Possum "cuticle" sample for a nitrogen analysis [see table 2.11]. The percentage recovery of nitrogen in the hydrolysate, for these two samples, was calculated [see table 2.13] and shows that there is a deficit of 7% in the Possum fur cuticle and 22% in

the Alpaca [brown] cuticle. Either the samples contain unidentified nitrogen compounds, or the recoveries are low due to hydrolytic losses.

The review by Rogers (1964), on the structure and biochemical features of the hair follicle, includes his observations of the occurrence of the amino acid citrulline in the inner root sheath of hair follicles and in medullary cells of hairs and quills.

In the hair follicle, the cuticle of the fibre lies in contact with the inner root sheath of the follicle [see figure 1.6] and it was thought that the cuticle may also contain citrulline. All the fibres [with the exception of wool] have a medulla and therefore citrulline will be present in their hydrolysates.

On running an authentic sample of citrulline [from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.], together with a Beckmann standard amino acid solution, it was found that citrulline was eluted with proline. Although they are eluted together, a quantitative result can be obtained for each amino acid. For higher accuracy however, a separation procedure was evolved [see section 2C (i) for both methods].

King (1967), after reinvestigation of the chromatograms from which the results of Bradbury, Chapman and King (1965a,b) were obtained, found that significant amounts of citrulline were present in all the wool cuticle hydrolysates. By increasing the normal column loading by ten to twenty times, he obtained

accurate values for the "total" citrulline in wool [0.073 mole %] and wool cuticle [0.481 mole %] hydrolysates. That citrulline has not been observed before in wool hydrolysates is understandable, since (a) the amount present is below the limit of detection with normal column loadings, (b) citrulline and proline are eluted at the same position, and (c) it was thought to be an unlikely constituent of proteins [Rogers (1964)].

Citrulline is present in hydrolysates of all the other fibres, as was expected, since they contained medullary cells. It was present in all the cuticle samples also, being particularly high in Possum "cuticle" which is due to the sample being contaminated with medullary cells [see later].

To see if the presence of pigment in the fibres would have any effect on the amino acid content, the two samples of Alpaca, one pigmented and the other unpigmented, were investigated. The amino acid composition of the fibres, apart from cysteic acid, tyrosine, phenylalanine and "total" citrulline is the same within experimental error [see table 2.5 and 2.6]. Cysteic acid is mainly due to oxidation of cystine caused by weathering [Bradbury, Chapman and King (1965a)], and therefore variable. Thus the only amino acids which are significantly different between the two fibres are tyrosine, phenylalanine and "total" citrulline.

The protein of the cuticles removed from their respective fibres, on the other hand, show significant differences for most of the amino acids. Threonine, serine, glutamic acid, glycine,

alanine, valine, histidine and arginine are the only amino acids not showing significant differences.

As tyrosine is a precurser for the formation of melanin [Fitzpatrick, Brunet and Kukita (1958)], it was expected that the tyrosine content in both the pigmented Alpaca fibre and cuticle would be considerably lower than in the unpigmented fibre and cuticle. However, this is not the case, there being a higher content of tyrosine in the pigmented Alpaca cuticle and fibre [although it was not significant in the fibre]. Apparently the supply of tyrosine for melanin formation is independent from that supplying the cells which form the fibre.

Comparison of the amino acid contents of all the fibres investigated, shows that wool and the two Alpaca fibres are the same, within experimental error, except for "total" citrulline and tyrosine. As stated earlier, the Merino wool contains no medulla and therefore will have a low citrulline content. The Alpaca fibres are medullated and it was therefore expected that citrulline would be present, which is in fact the case. Thus the only amino acid which shows a significant difference between wool and the two Alpaca fibres is tyrosine. The mammalian [Human hair] and marsupial [Possum fur] fibres, however, show some differences on comparison with the other fibres. Crewther et al. (1965) state that differences in amino acid content between Human hair and wool probably reflect a higher proportion of high-sulphur proteins in the Human hair. This is in accord with the known fact that Human hair has a higher matrix content

than most wool fibres [Crewther et al. (1965)]. The close similarity of composition of the widely differing fibre types must have some evolutionary significance.

Comparison of the amino acid composition of the cuticles, shows that the agreement is not as good as for the fibres. Apart from Possum fur "cuticle", the amino acid contents of them are similar, wool and the two Alpaca cuticles again showing close agreement.

The Possum fur "cuticle" sample has considerably higher contents of glutamic acid and citrulline and considerably lower contents of proline and arginine than the other cuticle preparations. It is known that medullary cells have very high glutamic acid and citrulline contents and low proline and arginine contents [Rogers (1962)]. Thus it appeared likely that the Possum fur "cuticle" sample could have been contaminated with medulla.

The observation of serrated cortical cells in the material dispersed from Possum fur [see section 2B (ii)], with the above analytical evidence, was clear proof that medullary cells were being dispersed from the fibre and contaminating the Possum fur cuticle sample.

Possum fur medullary cells were isolated by dissolving the fibre in alkali [see section 2B (iii)]. The amino acid composition of these cells is shown in table 2.15. Possum fur analysis is also included and in column three, the calculated composition of uncontaminated possum fur cuticle is recorded

TABLE 2.15

AMINO ACID ANALYSES [MOLE %] OF POSSUM FUR MEDULLARY CELLS, POSSUM FUR AND THE CALCULATED AMINO ACID COMPOSITION OF [PURE] POSSUM FUR CUTICLE[a]

Amino Acid	Possum Fur Medullary Cells	Possum Fur	Possum Fur Cuticle [Calculated]
Cysteic acid	0.21	0.23	0.25
Aspartic acid	4.98	6.31	3.15
Threonine	1.35	5.03	4.64
Serine	2.83	8.33	13.48
Glutamic acid	39.61	13.10	7.41
Proline	2.00	7.10	8.54
Citrulline	11.49	0.09	
Glycine	3.08	9.35	13.34
Alanine	3.17	6.12	5.25
Valine	2.56	5.93	5.79
Half cystine	0.15	11.65	20.13
Methionine	0.32	0.72	0.47
Isoleucine	1.39	3.22	1.96
Leucine	8.61	7.18	4.49
Tyrosine	1.59	3.20	2.32
Phenylalanine	2.07	1.89	1.33
Ornithine	7.47	0.47	
Lysine	6.01	3.09	3.51
Histidine	0.26	0.94	0.61
Arginine	0.82	6.05	3.21
Total citrulline	18.96	0.56	0.16
RAAA [%][b]	66.1	88.3	

[a] See text for method of calculation.

[b] Recovery of anhydroamino acids.

TABLE 2.16

AMINO ACID ANALYSES [MOLE %] OF RABBIT FUR MEDULLARY CELLS AND RABBIT FUR [FROM ROGERS (1962)]

Amino Acid	Rabbit Fur Medullary Cells	Rabbit Fur
Cysteic acid	-	-
Aspartic acid	4.26	5.62
Threonine	1.51	5.17
Serine	2.43	9.22
Glutamic acid	38.19	11.72
Proline	1.49	7.69
Citrulline	3.05	0.19
Glycine	2.50	8.12
Alanine	4.89	4.29
Valine	2.07	3.79
Half cystine	0	15.59
Methionine	0	0
Isoleucine	1.76	2.62
Leucine	14.00	7.22
Tyrosine	1.40	2.65
Phenylalanine	2.55	2.37
Ornithine Lysine	13.94[a]	3.99[a]
Histidine	0.80	2.17
Arginine	5.17	7.54
Total citrulline[b]	3.05	0.19
RAAA [%] [c]	71.3	72.6

[a] Sum of lysine and ornithine as two amino acids were not resolved.

[b] Minimal value as does not include citrulline converted to ornithine.

[c] Recovery of anhydroamino acids.

[see later]. For comparison, the composition of Rabbit fur medullary cells [isolated by the same method] and Rabbit fur, are shown in table 2.16 [from Rogers (1962)].

The low recovery of anhydroamino acids [66.1%] is similar to Roger's value [71.6%]. Only enough material for an amino acid analysis was obtained and therefore a nitrogen analysis was not performed on the cells.

There is very little agreement between the two analyses. Threonine, serine, glutamic acid, valine, isoleucine and tyrosine are the only amino acids showing little or no differences. However on comparing each medullary cell analysis with the fibre analysis, only four amino acids show large differences. In Rabbit fur, there is no difference in alanine content between the fibre and medulla, whereas in Possum fur, the alanine content of the fibre is about twice that of the medulla. The leucine content of the Possum medulla is not significantly higher than that of the fibre, but in Rabbit fur it is twice the value. Arginine in the Possum fur is eight times the value in the medulla, but in the Rabbit fur it is only 1.5 times the medulla value. Also there is considerably more citrulline in the Possum fur medulla than in the Rabbit fur medulla.

An estimation from the amino acid analysis, of the amount of medulla present, has been made. Let x be the fraction of medulla present in the "cuticle". Then (1-x) is the fraction of cuticle and therefore for any amino acid

$$c = ax + b\,(1-x)$$

Where a = value for the amino acid in [pure] medulla

b = value for the amino acid in [pure] cuticle

c = value for the amino acid in the "cuticle" sample analysed.

Assuming that the possum fur cuticle amino acid composition will be similar to the other cuticle compositions, an estimate of b can be made which leaves x the only unknown in the equation. Only those amino acids which show constancy throughout the cuticle samples [excluding the Possum "cuticle"] have been used for the calculation. The value used for b is the mean of the values from the four cuticle samples for the amino acids chosen. The calculated values for x are shown in table 2.17.

TABLE 2.17.

VALUES FOR FRACTION OF MEDULLA IN POSSUM "CUTICLE" SAMPLE.

Amino Acid	Values [mole %] for			x [Fraction of medulla]
	a	b	c	
Aspartic acid	4.98	3.42	3.60	0.115
Serine	2.83	12.38	10.82	0.163
Glutamic acid	39.61	8.66	15.46	0.220
Proline	2.00	10.93	6.91	0.451
Alanine	3.17	5.88	4.73	0.426
Leucine	8.61	5.14	5.52	0.110
Phenylalanine	2.07	1.33	1.52	0.256
			Mean	0.249

a. Values from table 2.15..
b. Average of values from tables 2.4, 2.5, 2.6 and 2.9.
c. Values from table 2.8.

Thus there appears to be about 25% of medullary cells in the cuticle sample.

Assuming that there is 25% medullary cells in the Possum cuticle sample and knowing the analysis of Possum medulla, b [the value for pure cuticle] is the only unknown in the above equation and thus can be calculated. The calculated composition of Possum cuticle is shown in table 2.15 [column 3]. These values must be considered as only being approximate; only analysis of an uncontaminated sample of cuticle will give the true composition.

The calculated composition of [pure] Possum fur cuticle resembles the Human hair cuticle composition, in that it has a very high cystine and lysine content and low arginine content [when compared with Merino wool cuticle]. The biggest differences are the low citrulline, alanine and valine contents. However, due to the large "total" citrulline content of the medulla, if the "total" citrulline content is calculated assuming 22% medulla contaminant, then the value for the cuticle would be 1.00 mole % which is closer to the "total" citrulline values in the other cuticle samples. This will make virtually no difference to the alanine and valine contents. Thus a value of 20% - 25% medulla contaminant is realistic.

The percentage differences of amino acids between cuticles and the parent fibres [except Possum fur] are shown in table 2.18. Since both the fibre and cuticle of wool and the two Alpaca fibres show remarkable agreement in amino acid content,

TABLE 2.18.

PERCENTAGE DIFFERENCES OF AMINO ACID CONTENT OF CUTICLE FROM PARENT FIBRE.

Amino Acid	Wool		White Alpaca		Brown Alpaca		Human Hair	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Cysteic acid	210		280		360		270	
Serine	27		21		23		0	
Proline	44		87		70		21	
Total citrulline	580		210		280		480	
Glycine	21		31		22		93	
Alanine	11		12		15		42	
Valine	33		39		56		65	
Cystine	61		62		25		23	
Aspartic acid		48		46		36		35
Threonine	24		22		16		44	
Glutamic acid	27		33		28		15	
Methionine	24		49		30		16	
Isoleucine	16		40		30		13	
Leucine	24		29		21		16	
Tyrosine	26		61		36		27	
Phenylalanine	41		52		50		23	
Lysine	0		20		0	50		
Histidine	13		0		14		43	
Arginine	25		30		27		58	

it follows that the percentage differences of the amino acids between cuticles and the parent fibres for these three fibres should be very similar. The results indicate that they are, apart from "total" citrulline, which is again due to wool not containing medullary cells. Apart from lysine, the differences for Human hair, between the cuticle and fibre, are also similar to the other fibres. No explanation can be suggested why the lysine content of Human hair cuticle is so large, giving the anomolous result.

The Merino wool has no medulla and therefore the wool cuticle sample can not be contaminated with medullary cells. The method of preparation of the cuticle would also preclude citrulline being absorbed in the protein. This can only imply that citrulline is a normal constituent of the cuticular protein. Similarly, for the other cuticle samples, the citrulline must be part of the polypeptide chains of the protein as, apart from Possum fur, the cuticle samples are not contaminated with medulla.

It is not known if the ornithine present in cuticle hydrolysates is all due to breakdown of citrulline during the acid hydrolysis, or is a constituent of the protein. King (1967), who has also analysed the cuticle from some keratin fibres, concluded that it is all formed during hydrolysis.

Mercer (1961) considers that the presence of citrulline in a protein may be diagnostic of trichohyalin, the protein of the inner root sheath. Initially, trichohyalin forms as amorphous droplets, but later is convered into a fibrous form. Medullary

cell protein also resembles trichohyalin [Rogers (1964), Mercer (1961)] and supports the above hypothesis. Since cuticle has an appreciable content of citrulline, is all or part of the cuticular protein trichohyalin?

Both of the above proteins [inner-root sheath and medullary cell] are non-keratinous and contain virtually no cystine. Mercer (1957) has stated that the endocuticle is non-keratinous. It can be seen in electron micrographs that the endocuticle is not stained as strongly as the exocuticle or rest of the fibre [e.g. see figure 3.6, a cross-section of a wool fibre stained with gold], which implies that the cystine content of the endocuticle is low. Happey and Johnson (1965) have concluded that in the follicle, the hair cuticle produces both amorphous and fibrous proteins in the same cell, similar to that produced in the inner-root sheath. Therefore, does the endocuticle resemble trichohyalin? If so, then the presence of citrulline in the cuticle can be understood. Only complete analysis of the endo- and exo-cuticle can answer this intriguing question.

Rogers (1964) suggests that citrulline is formed by desimination of arginine and that the ammonia [or a derivative] formed, may be used for amidation of side-chain carboxyl groups. Citrulline in cuticle could be formed in a similar manner or it could be incorporated in the protein after diffusion from the inner-root sheath.

Bradbury, Chapman and King (1965a) observed that the total amount of all the polar amino acids [aspartic acid, glutamic acid,

arginine, tyrosine, lysine, hystidine, threonine and serine] in wool cuticle, is lower than the total amount in wool, thus making the cuticle less polar than the parent fibre. Similarly, it can be seen from the analyses, that the other cuticles are also less polar than the parent fibre from which they were separated.

Wool cuticle has been shown by X-ray studies to be an amorphous protein [Woods (1938), Lustig, Kondritzer and Moore (1945)]. All the cuticle samples, except the Possum fur "cuticle", do not show birefringence when observed through a polarizing microscope with crossed Nicol prisms, thus indicating that no crystalline structure is present. In the case of the Possum fur cuticle sample, some birefringence is observed, presumably from the contaminating medullary cells.

Bradbury, Chapman and King (1965a) suggested that wool cuticle is amorphous due to its amino acid composition. Blout et al. (1960) and Blout (1962) have classified the amino acids according to their ability to produce synthetic homopolypeptides, which can have an α-helical structure in the solid state and in solution. It is found that all the amino acids which are enriched in the wool cuticle are non-α-helix forming [by the above classification], with the exception of glycine and citrulline which are unclassified. Of the amino acids which occur to a lesser extent in the wool cuticle, all, except arginine and histidine which have not been classified, isoleucine which is non-α-helix forming, and threonine which

is predicted to be non-α-helix forming, will form α-helical homopolypeptides. As the cuticles of the two Alpaca samples and the Human hair cuticle amino acid contents vary from the parent fibre composition in a similar manner to wool, it is therefore predicted [from their amino acid compositions] that these cuticles will also be amorphous. This appears to be the case, as these cuticle samples are non-birefringent. It appears that Possum fur cuticle, when the contaminating medullary cells are removed, will also have an amino acid composition similar to wool cuticle and thus will also be expected to be amorphous. However these results can not be confirmed conclusively until X-ray studies of the cuticles have been performed.

3. SEPARATION AND ANALYSES OF ORTHOCORTICAL AND PARACORTICAL CELLS OF WOOL.

[A] INTRODUCTION.

It was shown by Bradbury, Chapman and King (1965a), that the ultrasonic disintegration of wool [Bradbury and Chapman (1964)], avoids chemical modification of the protein in the fibre during the treatment. Thus, for the first time, a non-degradative method is available for isolation of histological components [cuticle and cortical cells] from wool and keratin fibres.

As the nature of the ortho- and para-cortices had not been resolved, it was decided to attempt to separate the two cortices, without any chemical modification, by using the above method.

At first it was hoped that after bilaterally dyeing the fibres, they could be disintegrated, giving a mixture of dyed

and undyed cortical cells. Some wool was bilaterally stained with Methylene Blue, Janus Green and Chicago Blue 6B, but the dyes were desorbed immediately the fibres were immersed in formic acid.

Some fibres were also bilaterally stained with metal ions [Ni^{++}, Cu^{++}, Co^{++}, Fe^{+++}], using the method of Corbett and Yu (1964). Again, immersion of the stained wool in formic acid caused the metal ions to desorb or spread evenly throughout the cortex.

Cortical cells, prepared by treating wool with ultrasonics [Bradbury and Chapman (1964)], were centrifuged on several different density gradients - chloroform/formic acid mixtures, aqueous chloral hydrate [Ward and Bartulovich (1955)] and aqueous sucrose - but no separation of the cortical cells was achieved. Apparently, the density of the two types of cortical cells is not very different, which is in contrast to cortical cells obtained by partial acid hydrolysis [Ward and Bartulovich (1955)] and by heating in water at 170°C [Horio et al. (1965)] [see section 1B(v)].

Some wool fibres which had been immersed in formic acid for over three years, were observed under the microscope. Quite surprisingly they were found to be partially separated into two segments. The wool was considerably modified as 36.5% had dissolved. Using a Zeiss microdissector equipped with glass microdissection needles, the two segments could be separated.

In an attempt to obtain wool which showed similar splitting

along its length, wool was refluxed in formic acid for various times. The formic acid was removed, any suspended material sedimented by centrifugation, and then freeze-dried to determine the amount of wool dissolved. Also, the fibres were examined microscopically for splitting. The results are shown in table 3.1.

TABLE 3.1.

PERCENTAGE DISSOLVED, AND MICROSCOPICAL OBSERVATIONS OF WOOL TREATED WITH FORMIC ACID.

1 gm wool treated in 50 ml formic acid.

Treatment	Amount Dissolved (%)	Microscopical Observation
Immersion 3 years	36.5	Cuticle removed. Splitting along length of cortex.
Reflux 30 min	7.3	Cuticle lifting.
Reflux 60 min	11.3	Cuticle partly removed. Some splitting of cortex.
Reflux 90 min	16.2	Cuticle removed. Increasing splitting with increasing time of treatment.
Reflux 2 hr	18.0	
Reflux 3 hr	22.0	
Reflux 18 hr	79.0	------

It was found that fibres which had been refluxed in formic acid for 1 hour, could be separated into two components using the microdissector. However, the method is extremely tedious and time consuming and was not pursued.

Fibres which have been refluxed for 30 min in formic acid can be dispersed completely by ultrasonics in a 30 min treatment. If after the reflux treatment, the formic acid is washed out and the fibres suspended in water and treated with ultrasonics, then some material is dispersed; about 10 to 20% being dispersed in a 30 min treatment. This is in contrast to the small breakdown of untreated wool by ultrasonics, when immersed in formic acid [6.8%] and water [0.1%] [Bradbury and Chapman (1964)].

It was found that fibres that had been refluxed in formic acid, even for 3 hours, could still be dyed bilaterally with Methylene Blue [using the conditions outlined by Davies (1963a) or Snyman (1963a)] or nickelous ions [using the method of Corbett and Yu (1964)]. When the bilaterally stained fibres, immersed in water, were treated with ultrasonics, only a very small amount of material was dispersed. Also, in the case of the fibres stained with Methylene Blue, dye tended to diffuse from the fibres.

Laxer and Ross (1954) reported that wool fibres could be bilaterally stained with gold, following a pretreatment in formic acid. As the staining procedure involves the use of formic acid, the gold apparently does not migrate in this media. This is in contrast to the other metal stains and dyes mentioned above.

Some wool, bilaterally stained with gold, was suspended in formic acid and treated in the ultrasonic disintegrator. Some material was dispersed and when examined microscopically,

two types of cortical cells could be seen, one dyed darker than the other. Also, fibres which had not been dispersed, were still stained bilaterally. Two layers [consisting of cortical cells] were obtained when the dispersed material was layered on a carbon tetrachloride-ethanol density gradient and centrifuged, one layer being darker in colour than the other.

This is the method outlined, by which ortho- and para-cortical cells were obtained. Amino acid analyses and high- and low-sulphur analyses were performed on the separated cortical cells and are reported below.

[B] EXPERIMENTAL

(i) Materials.

The wool used was the same Merino 64's quality as described in section 2B(i). Only A.R. 98-100% formic acid, redistilled until residue free, was used. All other chemicals were A.R. grade, unless otherwise stated.

The gold chloride solution was prepared as follows: 3 gm of chloroauric acid [$HAuCl_4.xH_2O$, B.D.H.,L.R.] was heated at 120°C for 12 hours to produce gold chloride [$AuCl_3$] [Garside and Phillips (1953)]. 2 gm $AuCl_3$ was dissolved in 100 ml distilled water to give a 2% solution, which was used for dyeing the wool.

(ii) Separation of Ortho- and Para-cortical Cells.

The wool was bilaterally stained with gold chloride by the method outlined by Laxer and Ross (1954), which is as follows:

1 gm of wool was immersed in 100 ml formic acid for 20 min, rinsed in distilled water for 2 min and then blotted dry on filter paper. The wool was placed in 100 ml of a 2% aqueous gold chloride solution for 10 min , rinsed again in distilled water and blotted dry. Finally, the fibres were immersed in 100 ml of 25% aqueous formic acid for 4 hours, and then either washed in distilled water [several changes over 3 to 4 days] or immersed in 50 ml formic acid [2 changes] ready for disintegration by ultrasonics.

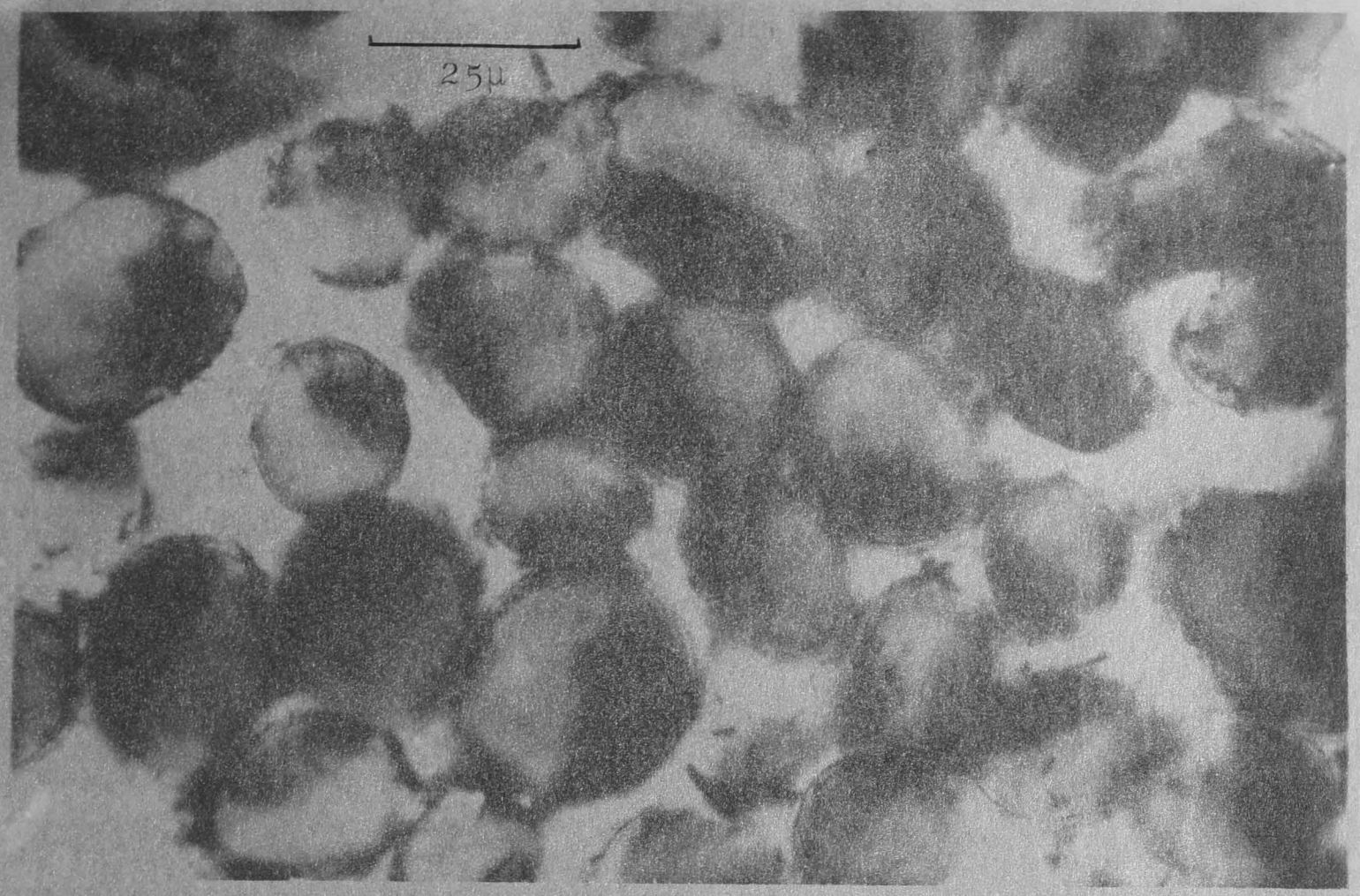


Figure 3.1. Light micrograph of a cross-section of wool fibres stained with gold.

Figure 3.2. Light micrograph of a wool fibre stained with gold.

A cross-section of some gold stained fibres is shown in figure 3.1, and figure 3.2 depicts a longitudinal view of a stained fibre. The stained segment is on the inside of the crimp wave, which indicates that the gold is concentrated in the paracortex. In the cross-section it can be seen that the stained area is smaller than the unstained area, which also indicates that the paracortex is the segment that has been stained. After supercontraction in water at 120°C, Laxer and Ross (1954) found that the unstained segment of the fibre was digested by trypsin, which confirms that the unstained segment is the orthocortex [Mercer (1953)]. This evidence, together with the evidence from electron microscopy [see section 3C] indicates that the densest deposit of gold is in the paracortex and the orthocortex, although it contains some gold, is the segment which is relatively unstained.

The wool was disintegrated in a 500 watt Mullard-MSE ultrasonic disintegrator [Bradbury and Chapman (1964)] using the following conditions:

The wool, immersed in 50 ml formic acid in a 300 ml round-bottomed beaker, was placed so that the 9:1 probe was half an inch from the bottom of the beaker. The beaker was surrounded by a stirred ice-water-salt cooling bath and the instrument tuned to maximum cavitation at full power. The wool was treated for two hours.

After the ultrasonic treatment, the dispersed material was separated from the remaining fibres by pouring the suspension

through a stainless steel sieve [35 mesh]. The suspension of cuticle and cortical cells was sedimented by centrifugation and the formic acid removed. The sedimented material was washed 6 or 8 times with ethanol, centrifuging between each washing. The cortical cells were freed of cuticle, and any short lengths of fibres by the sieving process of Bradbury and Chapman (1964). After pouring the ethanolic suspension through a 120 micron [pore size] Nytrel filter cloth to remove the wool fibres, the suspension was poured onto a 50 micron Nytrel filter cloth; the cortical cells were trapped on the cloth and freed of cuticle which passed through.

The cortical cells [in ethanol] were checked for purity by light microscopy and were found to contain no cuticle or wool fibres. Cells differing in intensity of stain could be seen. The fibres remaining after the ultrasonic treatment were also checked, and found to be still bilaterally stained.

Separation of the ortho- and para-cortical cells was accomplished using carbon tetrachloride-ethanol density gradients, which were formed using the simple apparatus shown in figure 3.3. The apparatus is based on figure 6A in Oster and Yamamoto (1963). Tubes A and B [Pyrex test tubes 20 mm by 200 mm] are connected by a teflon stopcock C. The outlet, D, consists of a removable PVC tube that has been drawn to a fine diameter. The size of this tube controls the rate of flow of liquid from the apparatus. The flow rate must be fairly slow [5 to 10 ml per min] to achieve a linear gradient. E is a glass capillary tube connected to a nitrogen supply.

[For reproducible results the nitrogen must be saturated with the liquid forming the gradient, by passing through a bubbler immersed in the liquid].

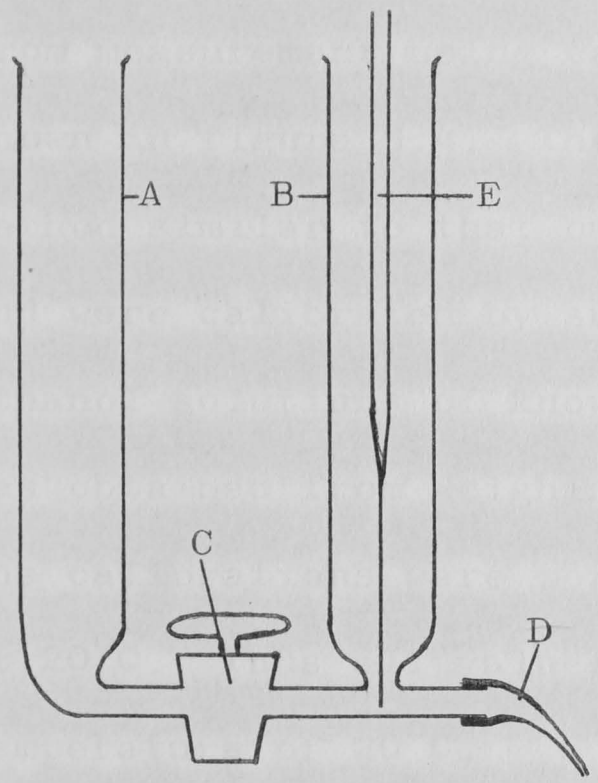


Figure 3.3. Apparatus used to produce density gradients.

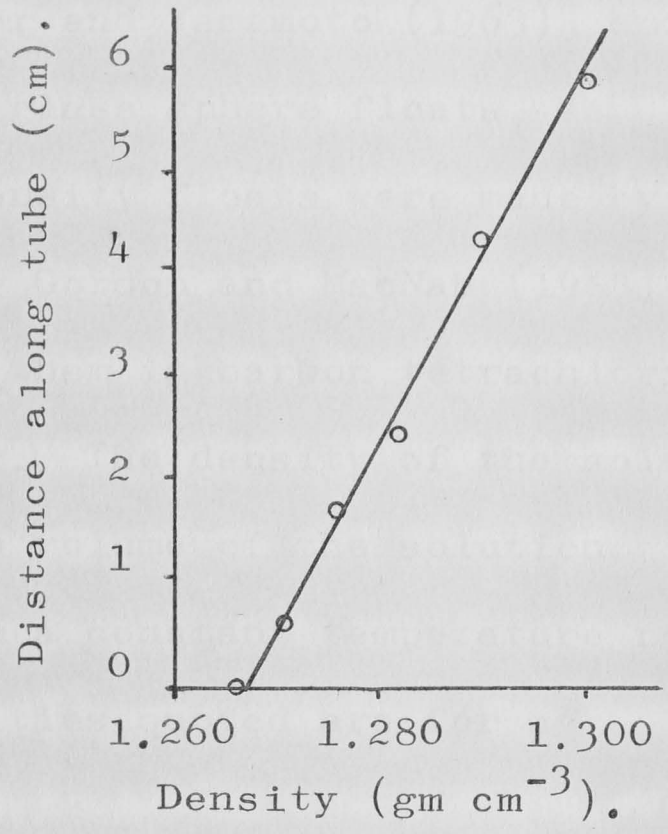


Figure 3.4. Plot showing linearity of gradient.

The operation of the apparatus is as follows:

After arranging a 40 ml centrifuge tube under the outlet D, so that the tip of the outlet tube is in contact with the side of the centrifuge tube, the tap C is turned to the "off" position. 15 ml of the low density liquid is introduced into tube A by means of a syringe and the nitrogen supply adjusted so that a steady stream of nitrogen issues from the capillary tube. 15 ml of the high density liquid is quickly introduced into tube B, and tap C turned to the "on" position. As the liquid from tube A flows into tube B, it is mixed with the liquid in

tube B by the nitrogen from the capillary. Thus the liquid issuing from D becomes progressively less dense.

The gradient produced by this apparatus is linear, both from theoretical predictions [Oster and Yamamoto (1963)] and from measurement using calibrated glass sphere floats, as shown in figure 3.4. The glass density floats were made by a method similar to that outlined by Gordon and MacNab (1953) and were calibrated by suspending them in carbon tetrachloride-ethanol solutions of known density. The density of the solutions was obtained from weighing a known volume of the solution. The calibrations were performed in a constant temperature room at $20^{o}C$. Thus all values of densities quoted are for a temperature of $20^{o}C$.

Although a single gradient could have been used to separate the two cortices, it was decided to use two gradients to achieve least contamination of both species. A number of gradients of density 1.265 to 1.300 gm cm^{-3} were made, the ethanolic suspension of cortical cells layered on top [3 ml of the suspension containing about 4 mgm of cortical cells, per tube] and then centrifuged at 3,500 r.p.m. [relative centrifugal force (RCF), 1,200G] in a swing-out rotor at $20^{o}C$. It was found necessary to continue centrifugation for 16 to 20 hours to achieve adequate separation.

The top layer of each centrifuge tube containing the lighter orthocortical cells [density 1.272 to 1.275 gm cm^{-3}, obtained from the calibrated glass sphere floats] was removed

using a Pasteur pipette and washed several times with ethanol, prior to drying at room temperature under vacuum for 24 hours. The sedimented material containing the paracortical cells was also washed several times with ethanol before placing on top of gradients of density 1.290 to 1.340 gm cm^{-3}. Again these were centrifuged for 16 to 20 hours. The layer sedimenting at a density of 1.314 - 1.316 gm cm^{-3} was recovered as above and consisted of the heavily stained paracortical cells. The cells were washed in ethanol and dried under vacuum at room temperature. A small amount of material which sedimented to the bottom consisted of glass chips, titanium from the ultrasonic disintegrator probe and a few very heavily stained cortical cells.

From 1 gm of stained wool, 58.0 mgm of orthocortical cells and 26.3 mgm of paracortical cells were obtained. As all the wool was not dispersed, the above values do not necessarily indicate the ratio of the cortices in the fibre. Analyses of the fibres remaining after the ultrasonic treatment, showed that they contained a higher percentage of gold [16.7%] than the wool before the treatment [9.65%]. This indicates that the orthocortical cells are dispersed at a greater rate than the paracortical cells and thus the remaining wool is enriched in paracortical material. When the wool residue was microscopically examined, it was found that in most of the fibres the undyed orthocortical cells were missing, leaving the stained paracortex relatively intact.

Some cortical cells from unstained wool were obtained by the ultrasonic method as outlined above. After layering on a carbon tetrachloride gradient and centrifuging for 16 hours, they were found to sediment at a density of 1.280 gm cm^{-3} [obtained from the calibrated glass sphere floats]. Thus the observed density of the orthocortical cells [1.274 gm cm^{-3}] is lower than was expected, since they contain some gold.

The densities that were expected, have been calculated assuming that (a) the density of the cortical cells would be 1.280 gm cm^{-3} and (b) the gold content is equal to the ash content of the cells [see section 3D(i)]. The results are shown in table 3.2 and indicate that the orthocortical cells have swollen 3.5% and the paracortical cells 3.6%, due to the presence of the gold.

TABLE 3.2.

DENSITIES AND GOLD CONTENTS OF ORTHO- AND PARA-CORTICAL CELLS.

Material	Density Observed (gm cm^{-3})	Density Calculated (gm cm^{-3})	Gold Content[a] (%)
Orthocortical cells	1.274	1.318	3.11
Paracortical cells	1.315	1.362	6.44

[a] Ash analyses of cells.

(iii) Preparation of High- and Low-sulphur Proteins.

The method is a combination of that used by Crewther et al. (1966) for the isolation of low-sulphur proteins from keratin fibres, and the method used by Gillespie and Inglis (1965) for the isolation of high-sulphur proteins from the same fibres. These methods are based on the work of Harrap and Gillespie (1963). The method is as follows:

(a) Approximately 50 mgm of protein was dried under vacuum for 1 hour at 100°C, cooled and weighed. The protein was then extracted at 40°C for 3 hours in a glass stoppered test tube, with 2 ml of a solution 8M in urea and 0.2M in potassium thioglycollate, at pH 11.0. The test tube was fixed to a mechanical shaker mounted over a water bath, enabling the test tube to be agitated while immersed in the bath to ensure maximum extraction. The urea-thioglycollate solution was prepared by adding to a 0.4M potassium thioglycollate solution at pH 11.0 [made by adding KOH to thioglycollic acid to pH 11.0], urea and water, to give the desired concentration of reagents.

(b) The extracted proteins from the untreated wool were separated from the residue by filtration under vacuum through a tared No. 4 sintered glass crucible [Harrap and Gillespie (1963)]. The residues from the wool stained with gold, and the ortho- and para-cortical cells, were separated by centrifuging in a tared 6 ml centrifuge tube at 10,000 r.p.m. [RCF, 13,000 G] for 1 hour [Crewther et al. (1966)]. The residues were washed with several changes of water, dried at 110°C and weighed.

(c) The supernatant from (b) was alkylated by adding 0.40 gm iodoacetic acid and 0.30 gm tris(hydroxymethyl)aminomethane for each 5 ml of extract. After 15 min, the unreacted iodoacetate was destroyed by addition of potassium thioglycollate until in excess. When a small drop of the solution placed on a filter paper containing sodium nitroprusside gave a pink colour, then there was an excess of thioglycollate. The filter paper was prepared by dipping it into a fresh 2% aqueous sodium nitroprusside solution and then drying.

(d) The alkylated solution was quantitatively transferred to Visking cellulose casing dialysis tubing and dialysed against distilled water for two days, with at least six changes of distilled water.

(e) To the dialysed extract in a 50 ml [cellulose nitrate] centrifuge tube, an equal volume of pH 4.4 sodium acetate buffer of ionic strength 0.8 was added. The low-sulphur proteins that were precipitated were sedimented by centrifuging for 1 hour at 10,000 r.p.m. [RCF, 16,000 G].

(f) The supernatant was quantitatively transferred to Visking cellulose casing dialysis tubing and dialysed against distilled water [at least 6 changes] for two days. After dialysis, the solution was freeze-dried in a tared flask to give the HIGH-SULPHUR [SCMKB] proteins.

(g) The sedimented low-sulphur proteins from (e) were redissolved in pH 9 saturated aqueous sodium tetraborate [borax] buffer by gentle agitation on a shaking machine. The low-sulphur proteins

were again precipitated with pH 4.4 sodium acetate buffer [ionic strength 0.8] and sedimented by centrifugation to free them of any occluded high-sulphur proteins. The supernatant was discarded and the sedimented protein dissolved in the saturated borax buffer. The solution was filtered through a No. 4 sintered glass funnel to remove foreign material [from the buffer solutions]. The filtrate was quantitatively transferred to Visking cellulose casing dialysis tubing and dialysed against distilled water [at least 6 changes] for two days. The dialysed solution was then freeze-dried in a tared flask to give the LOW-SULPHUR [SCMKA] proteins.

[C] ELECTRON MICROSCOPY

The wool fibres, after being bilaterally stained with gold, were aligned and embedded in Araldite [Glauert, Rogers and Glauert (1956)]. Cross-sections were prepared with a Servall Porter-Blum ultramicrotome using a diamond knife, picked up on grids coated with formvar and carbon, and examined in a J.E.M., model T6, electron microscope.

At low magnifications, most of the gold appears to be deposited as large spherical particles in the nuclear remnants of the paracortical cells [see figure 3.5]. However, at higher magnification smaller spherical gold deposits can be seen dispersed about the remainder of the cortical cells, as shown in figures 3.6 to 3.8. The cell membranes have not been stained at all and appear as light lines against the darker stained background. This is not surprising as it is known that formic acid extracts cell membranes from wool [Bradbury, Chapman and King (1965b)].

Laxer and Ross (1954) report that the background red colour in the fibres can be removed by continually flooding the fibre for a few hours with a 1% potassium cyanide solution at room temperature. After the treatment, all that could be seen were black streaks of gold in the cortex, and in cross-sections, black dots. These were apparently the heavily stained nuclear remnant areas in the paracortex.

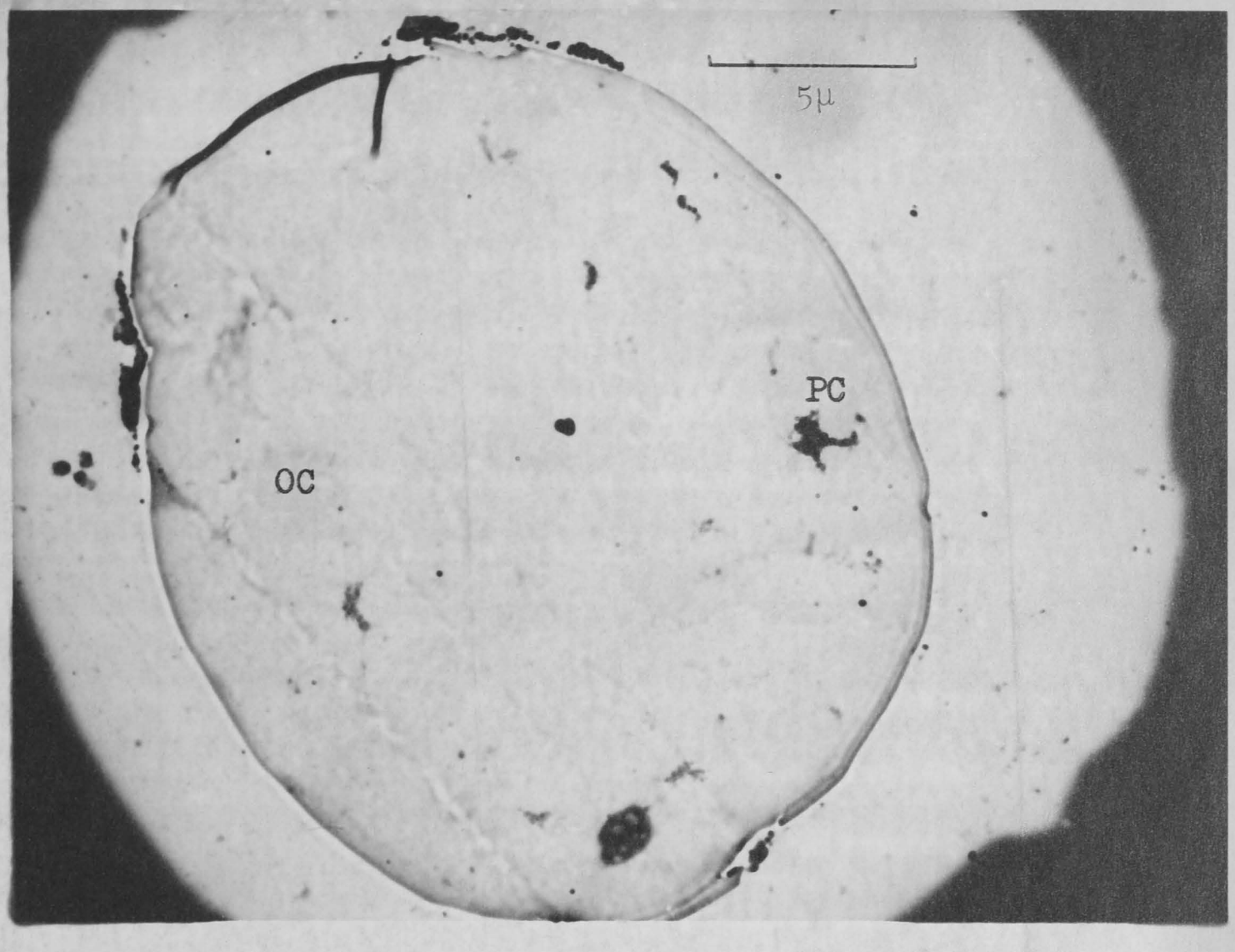


Figure 3.5. Electron micrograph of a cross-section of a wool fibre stained with gold.

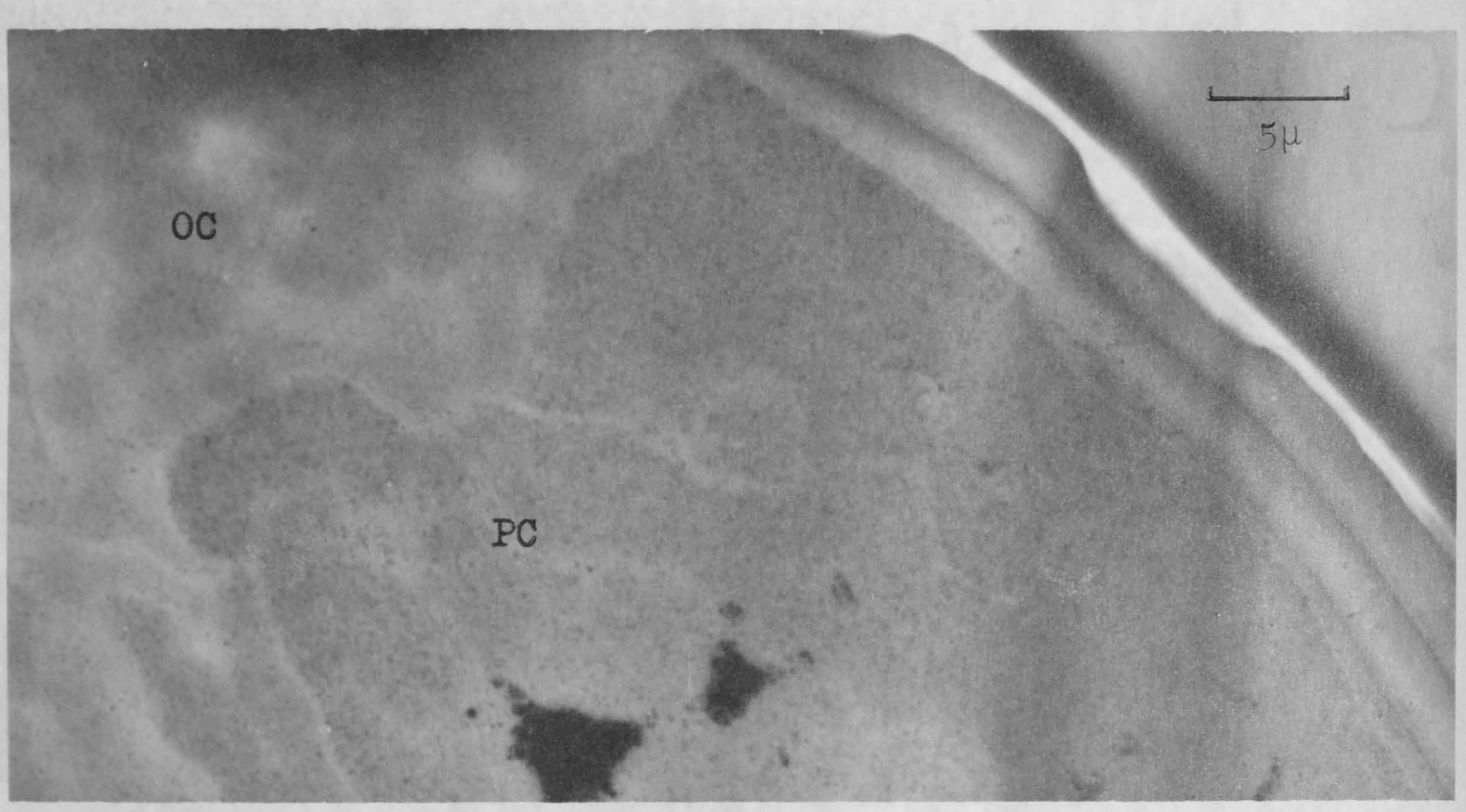


Figure 3.6. Electron micrograph of part of a cross-section of a wool fibre stained with gold.

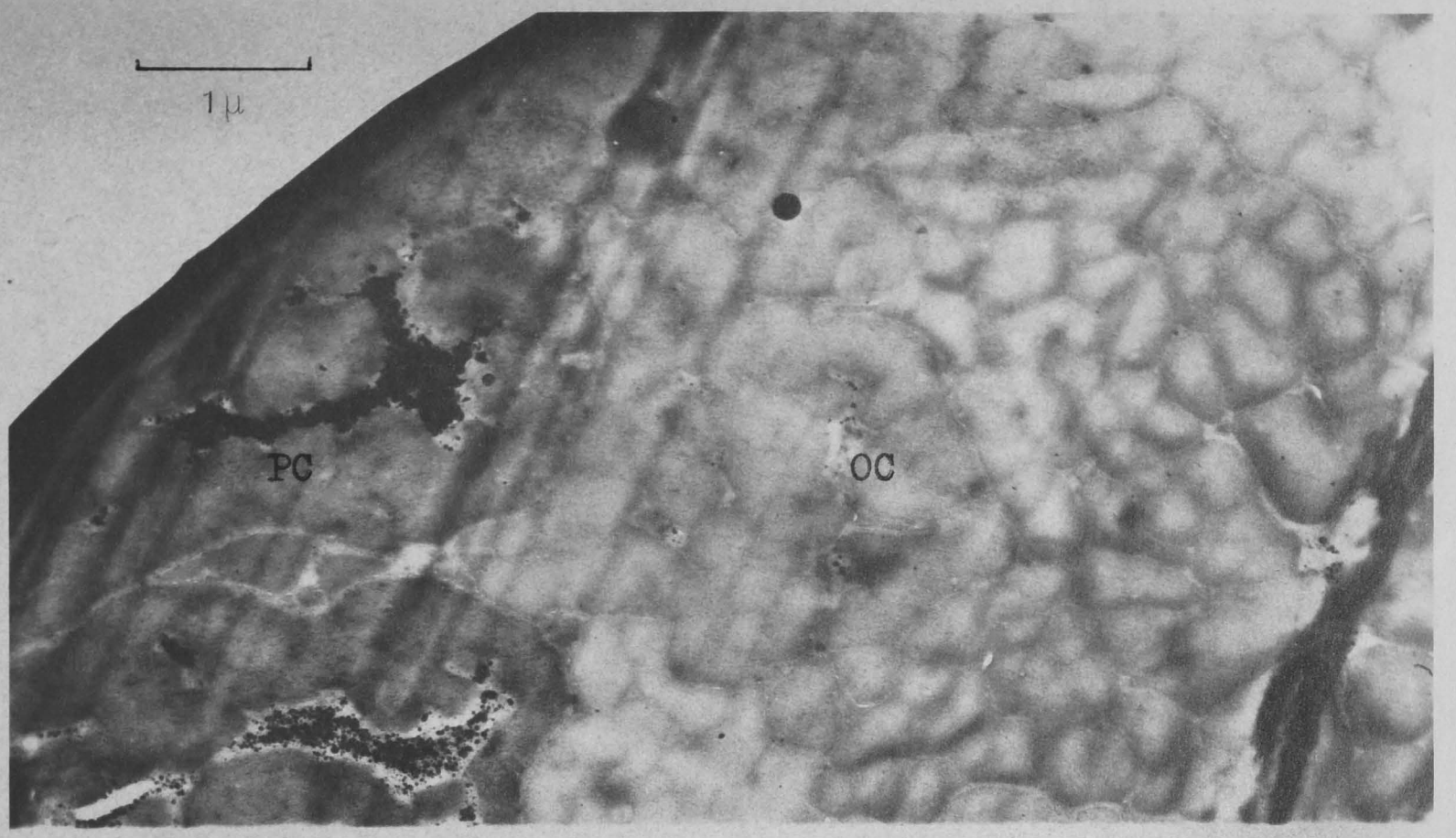


Figure 3.7. Electron micrograph of part of a cross-section of a wool fibre stained with gold, and post-stained with $KMnO_4$.

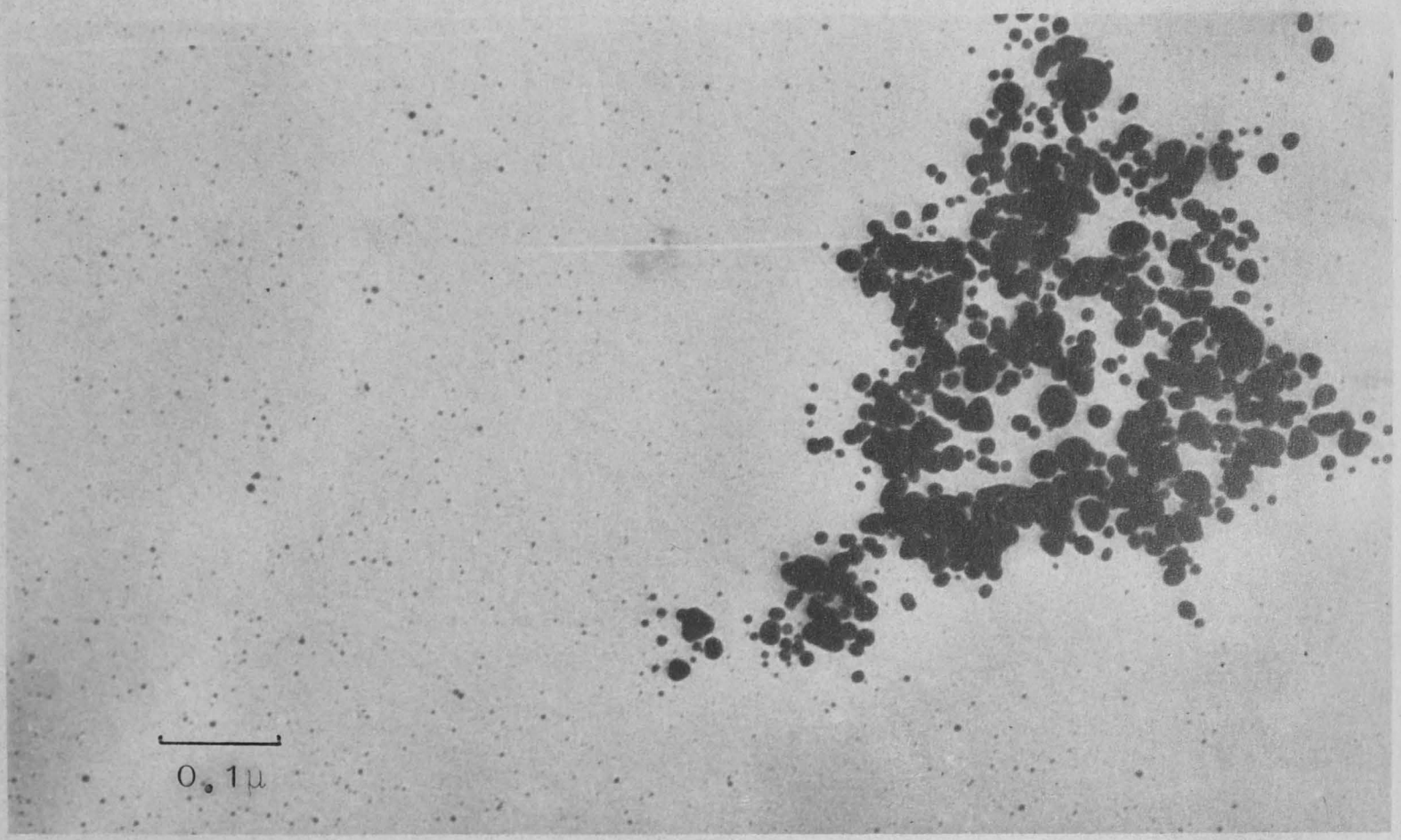


Figure 3.8. High magnification electron micrograph of the paracortex of a wool fibre stained with gold.

There is no doubt that the heavier deposit of gold resides in the paracortex. This was observed in numerous sections of which figure 3.5 is typical. Figure 3.6 is at the junction of the orthocortex [OC] with the paracortex [PC], and here it is very evident that the staining is more intense in the paracortex than in the orthocortex.

That the more intense staining is in the paracortex becomes more evident when the sections are post-stained with potassium permanganate [Rogers and Filshie (1962)], as shown in figure 3.7. The paracortex [PC] can be easily identified by its different appearance compared to the orthocortex [OC].

As can be seen in figure 3.6, the endocuticle has heavier deposits of spherical gold particles than the exocuticle, but the exocuticle is more evenly stained and darker than the endocuticle. The size of the spherical gold particles have been estimated from figure 3.8, and range from 100 Å [0.01 microns] to 400 Å [0.04 microns] in the nuclear remnants. Gold particles in the cortex [outside the nuclear remnant boundaries], range in size from 20 to 80 Å, with most of the particles between 30 Å and 40 Å in diameter.

Mellor (1923) reports that the size of particles in a purple-red gold solution range from about 350Å to 750Å in diameter. Thus it would be expected that the wool would be coloured a reddish-purple due to the gold, and this is in fact the colour of the gold-stained wool.

It was noticed that after the fibres were stained with

gold chloride, and when immersed in the 20% formic acid solution, that not only did the fibre turn red due to deposition of colloidal gold, but the aqueous formic acid solution also became red. Apparently some of the gold chloride had been reduced to gold by the formic acid. Addition of a gold chloride solution to 20% formic acid however, remained clear; no colloidal gold was formed.

From the amino acid analysis of the gold-stained wool [see section 3D(ii)], it appears that only about 50% of the gold chloride which diffuses into the fibre, is reduced to metallic gold by oxidation of cystine. The other 50% of the gold is apparently reduced by the formic acid.

The reduction of gold by cystine should produce a histochemical deposition of gold similar to that observed with osmium or silver staining of wool, and should not produce the large spherical particles of gold as are observed. That the spherical particles of gold are probably superimposed on a lightly stained background cannot be overlooked and would offer an explanation for the two methods of reduction. The gold reduced by cystine would give an overall light staining, but as the amount of gold reduced is not enough for intense electron staining, no structure can be seen in the background of figure 3.8. This would indicate that the darker and more even staining of the exocuticle, compared to the endocuticle, is due to a much higher cystine content. Secondly, the large gold particles deposited at random, will be due to reduction of the gold chloride by the formic acid.

It has been shown by Bradbury, Chapman and King (1965b), that the nuclear remnants and cell membrane material of wool is extracted by formic acid. In the 20 minute immersion in formic acid prior to staining with the gold chloride solution, the nuclear remnant and cell membrane material of the wool fibres would be extracted, leaving comparatively large empty volumes within the fibres. On diffusing into the fibre, high concentrations of gold chloride would be expected to collect in these areas and when reduced give large deposits of gold. This is also probably the explanation for the larger sized particles in the nuclear remnant material, compared to those in the remainder of the cortex.

The reason for the higher content of gold particles in the paracortex compared to the orthocortex, has not been elucidated. However, a possible explanation is as follows. Rogers and Filshie (1963) and also Kassenbeck (1965b) conclude that the ratio of filaments to matrix is about 1:1 in the paracortex and about 4:1 in the orthocortex. This means that there is considerably more matrix in the paracortex than in the orthocortex. Bendit (1966) has found that formic acid mainly penetrates the matrix regions of the wool cortex, swelling them 250% - 300%, whereas the filamentous regions are relatively unswollen and unaltered. Thus the site of nucleation of the gold particles would be expected to be in the matrix regions of the cortex, since this is the site of the formic acid. Also, as there is more matrix in the paracortex than the orthocortex,

one would expect a higher density of gold particles in the paracortex compared with the orthocortex. This is in fact the case.

[D] RESULTS AND DISCUSSION

(i) Chemical Analyses.

The chemical analyses reported include high- and low-sulphur protein analyses and gold analyses.

In wool, the mineral material [ash] appears to be concentrated in the cuticle. Bradbury and King (1967) have concluded that there is 10% cuticle in Merino wool and on inspection of table 2.11, it can be seen that the ash in wool [0.22%] can all be accounted for by the ash in the cuticle [2.27%].

Therefore, since there is no mineral material [ash] in the cortical cells, the ash content of the cortical cells containing gold will be equivalent to the amount of gold in the cells. This will also apply to proteins extracted from them. However, for the gold-stained wool, 0.22 must be subtracted from the percentage of ash to give the true gold content of the fibres. The gold contents [based on ash analyses] of gold-stained wool, ortho- and para-cortical cells, and their low-sulphur proteins, are shown in table 3.3.

It can be seen that there is less gold in the isolated cortical cells than would have been expected from the gold content of the fibre. During the disintegration, it was noticed that the formic acid became coloured dark purple-red with colloidal gold. Also, the cuticle was stained a deeper colour

than the cortical cells. Thus the remainder of the gold is in these two fractions which were not analysed.

Using the extraction method described by Harrap and Gillespie (1963) [0.2M potassium thioglycollate in 8M urea at pH 11], Gillespie (1964) and Gillespie, Reis and Schinckel (1964) were able to obtain 80% to 92% extraction of proteins from various Merino wools. The method of estimation is not stated, but is probably by measurement of the refractive index increment of the dissolved proteins, in which an assumption for the value of dn/dc has to be made [see Harrap and Gillespie (1963)] This would not seem to be as satisfactory as the method adopted in this investigation [see section 3B(iii)], where the proteins are dialysed, freeze-dried and weighed. This avoids any assumptions and therefore is more precise.

TABLE 3.3.

GOLD CONTENTS[a] OF STAINED WOOL, WOOL SEGMENTS AND ISOLATED LOW-SULPHUR [SCMKA] PROTEINS

Protein	Gold Content (%)
Gold-stained wool	9.43[b]
Orthocortical cells	3.11
Paracortical cells	6.44
SCMKA from gold-stained wool	10.5
SCMKA from orthocortical cells	3.7
SCMKA from paracortical cells	9.5[c]

[a] Obtained from ash analyses: See text.

[b] True ash analyses 9.65%.

[c] This value could be in error as sample for analysis was not large enough for an accurate value.

The extraction method outlined here, is also the same as that used by Harrap and Gillespie (1963). However, the average amount of protein extracted in five analyses was 70.7% with a maximum of 74.7%. Thus the results reported by Harrap and Gillespie (1963), Gillespie (1964) and Gillespie, Reis and Schinckel (1964), could not be repeated during this investigation. This possibly indicates that the value they assumed for dn/dc is in error.

It was found that after extraction of the samples containing gold, all the gold in the solution could not be sedimented, even when centrifugation was prolonged for several hours. Apparently the proteins had formed a protective colloid around some of the finely divided gold, which prevented it from sedimenting. It was found that the gold which remained in solution, was sedimented with the low-sulphur proteins, colouring them red. The high-sulphur protein fraction, when freeze-dried, was white and thus contained no gold. Gold [ash] contents of the low-sulphur proteins were obtained [see table 3.3], but ash analyses were not performed on the residues, as they could not be recovered.

The contents of high-sulphur proteins, low-sulphur proteins and residue of the wool and cortical cells is shown in table 3.4, where it can be seen that apart from the analysis of the paracortical cells, all of the material in the samples is accounted for, within experimental error. The reason for the low recovery of material from the paracortical cells could be

TABLE 3.4.

CONTENT OF HIGH-SULPHUR AND LOW-SULPHUR PROTEINS AND RESIDUE IN WOOL AND CORTICAL CELLS.

All results are based on dry weight.

Material	Weight[a] (mgm)	High-S Proteins (%)	Low-S Proteins (%)	Residue (%)	Total (%)
Wool	50.2	17.7	50.0	31.9	99.6
Gold-stained wool	48.4	27.9	49.0[b]	19.2[b]	96.1
Orthocortical cells	42.4	47.6	40.8[b]	8.3[b]	96.7
Paracortical cells	15.05	44.5	34.6[b]	7.3[b]	86.4

[a] Dry weight of material analysed.

[b] These components contained gold.

due to inadequate experimental technique in handling the small amount of material available for the analysis, or could be due to losses from small polypeptides able to diffuse through the dialysis bag. It is not known whether there was a large loss of material from only one component, or an even loss of material from two or all three components. Unfortunately, lack of more paracortical cells and time prevented a repeat analysis.

If an allowance is made for the amount of gold in the low-sulphur proteins and residue [calculated from the total

gold content in the sample and from that in the low-sulphur proteins], and the total percentage of material accounted for is taken into account, then the percentages of the three components in the "pure" sample can be calculated and are shown in table 3.5. Also, the results obtained from the untreated wool sample and results obtained by Gillespie, Reis and Schnickel (1964) are included.

TABLE 3.5.

THE CONTENT OF HIGH-SULPHUR PROTEINS, LOW-SULPHUR PROTEINS AND RESIDUE IN WOOL AND CORTICAL CELLS.

All results expressed as percentage of dry weight.

Material	High-S Proteins	Low-S Proteins	Residue
Wool [Gillespie, Reis and Schinckel (1964)]	22[a]	68[b]	10[b]
Wool	17.8	50.2	32.0
Wool [Gold-stained][c]	32.0	50.4	17.6
Orthocortical cells[c]	50.9	41.8	7.3
Paracortical cells[c,d]	55.0	38.7	6.3

[a] Average of 14 determinations.

[b] These values are not stated but are implied: See text.

[c] Calculated: Values give contents in gold-free samples.

[d] Results possibly inaccurate: See text.

Gillespie, Reis and Schinckel (1964) only quote values for the amount of material extracted and the percentage of high-sulphur proteins in the wool. As mentioned previously, the amount of protein extracted was apparently estimated from a measurement of the refractive index increment. Also the method of determining the amount of high-sulphur proteins is not stated, but was probably estimated by the Biuret reaction which involves, using as a standard, a previously isolated sample of high-sulphur proteins [see Gillespie (1964)]. This would not appear to be as satisfactory as freeze-drying and weighing the high-sulphur proteins, as used in this investigation [see section 3B(iii)]. Further, the amount of residue and low-sulphur proteins are not stated, but are implied from the above values. It appears that direct analyses of the amount of low-sulphur proteins and residue have not been performed previously, as there is no record of any such analyses in the literature.

The results obtained for the untreated wool and the gold-stained wool, show surprisingly good agreement in their content of low-sulphur proteins, as shown in table 3.5. This must imply that the method is reproducible. The difference in the contents of high-sulphur proteins and residue, indicate that in the unstained wool sample, some of the high-sulphur proteins remained undissolved. Why oxidation of some cystine to cysteic acid should increase the amount of high-sulphur proteins extracted cannot be explained.

The above results for wool do not agree with the results obtained by Gillespie, Reis and Schinckel (1964), and could be due to the different method of estimating the components. The above authors found that the high-sulphur protein content of 14 Merino wools ranged from 21% to 24% [average 22%] and therefore the Merino wool sample used in this investigation would not be expected to give such vastly different results.

The ratio of low-sulphur to high-sulphur proteins in the orthocortical cells, is almost 1:1 [see table 3.5], which is a surprising result. It has been thought that the low-sulphur proteins are derived from the filamentous regions of the fibre and the high-sulphur proteins from the matrix regions [see Crewther et al. (1965)]. Rogers and Filshie (1963) and Kassenbeck (1965b) have concluded that the ratio of filaments to matrix is 4:1 and not less than 4:1 respectively. It was therefore expected that the ratio of low-sulphur proteins to high-sulphur proteins would be about 4:1.

The result, that approximately equal amounts of the high- and low-sulphur proteins were extracted from the orthocortical cells, must cast doubt on the validity of the above hypothesis that the high-sulphur proteins form the matrix material in the fibre and the low-sulphur proteins the filaments.

Other evidence has been found recently which also casts doubt on the hypothesis. Bendit and Feughelman (1967) have suggested that the low-sulphur proteins extracted from wool, are not only derived from the filaments, but may also form part of

the matrix. Filshie (1967) also suggests that the filaments may have a high-sulphur protein component, since, in high resolution electron micrographs of cross-sections of wool fibres, densely stained threads can be seen protruding from some filaments into the surrounding matrix. The idea of the low-sulphur proteins being derived entirely from the filaments and the high-sulphur proteins from the matrix thus appears to be false. It appears reasonably certain from electron micrographs, that the ratio of filaments to matrix in the orthocortex is much higher [4:1] than in the paracortex [1:1]. Thus, in the orthocortex, there must be considerable amounts of high-sulphur proteins in the filaments.

The results obtained for the paracortex, also indicates about a 1:1 ratio of high-sulphur proteins to low-sulphur proteins. However, there is some doubt as to the validity of the values quoted in table 3.5, due to the low amount of material accounted for [86.4%].

(ii) Amino Acid Analyses.

After hydrolysis of the proteins, amino acid analyses were performed using the Technicon amino acid analyser equipped with two ion-exchange columns, as described in section 2C(i). All results reported are the average of duplicate analyses and are quoted in mole per cent. The recovery of anhydroamino acids [RAAA] is also recorded for each analysis. The remarks concerning amino acid analyses in section 2C(i) and 2C(ii) apply equally to these analyses.

For comparison between analyses, total half cystine contents are also included in the tables. For the gold-stained wool, orthocortical cell and paracortical cell analyses, this is the sum of cysteic acid and half cystine contents. For the high- and low-sulphur proteins, the total half cystine content is the sum of cysteic acid, half cystine and SCM-cysteine [S-carboxymethylcysteine].

In the hydrolysates of the high- and low-sulphur proteins, a small amount of cystine was found. This is apparently formed during hydrolysis from breakdown of the SCM-cysteine [Gillespie, Reis and Schinckel (1964)]. There does not appear to be a constant fraction of the SCM-cysteine converted to cystine, as the amount ranges from 4.4 to 19.7% of the total SCM-cysteine in the samples.

The amino acid composition of wool and gold-stained wool is shown in table 3.6. The wool analysis is slightly different to the wool analysis shown in table 2.4, which was taken from Bradbury, Chapman and King (1965a). The wool used for obtaining the ortho- and para-cortical cells was a different sample to that used for the analysis shown in table 2.4, although from the same sheep, but from a different shearing. Comparing the two analyses, it can be seen that they agree [within experimental error] apart from proline and methionine, proline being higher and methionine lower in this sample of wool.

Table 3.7 shows the amino acid composition of the orthocortical cells, paracortical cells, and for comparison that

TABLE 3.6.

AMINO ACID ANALYSES [MOLE %] OF WOOL AND GOLD-STAINED WOOL.

Amino Acid	Wool	Gold-Stained Wool
Cysteic acid	0.06	1.56
Aspartic acid	6.52	6.56
Threonine	6.63	6.41
Serine	10.69	10.59
Glutamic acid	12.18	12.63
Proline	6.77	6.86
Glycine	8.33	8.23
Alanine	5.33	5.57
Valine	5.51	5.81
Half cystine	9.99	7.78
Methionine	0.38	0.40
Isoleucine	3.08	3.22
Leucine	7.61	7.58
Tyrosine	3.89	3.46
Phenylalanine	2.72	2.74
Lysine	2.97	2.94
Histidine	0.87	0.92
Arginine	6.48	6.73
Total 1/2 Cys[a]	10.05	9.34
RAAA [%][b]	97.0	86.6

[a] Sum of half-cystine and cysteic acid.

[b] Recovery of anhydroamino acids.

TABLE 3.7.

AMINO ACID ANALYSES [MOLE %] OF ORTHOCORTICAL CELLS, PARACORTICAL CELLS AND GOLD-STAINED WOOL.

Amino Acid	Orthocortical Cells	Paracortical Cells	Gold-Stained Wool
Cysteic acid	2.69	2.69	1.56
Aspartic acid	7.66	7.36	6.56
Threonine	6.23	6.68	6.41
Serine	10.08	10.24	10.59
Glutamic acid	12.11	13.10	12.63
Proline	6.15	6.72	6.86
Glycine	9.29	8.01	8.23
Alanine	5.99	5.75	5.57
Valine	5.62	5.76	5.81
Half-cystine	4.87	6.04	7.78
Methionine	0.08[a]	0.11[a]	0.40
Isoleucine	3.20	3.44	3.22
Leucine	8.68	7.75	7.58
Tyrosine	3.63	2.88	3.46
Phenylalanine	3.10	2.77	2.74
Lysine	2.73	2.93	2.94
Histidine	0.78	0.89	0.92
Arginine	7.10	6.87	6.73
Met. sulphone[b]	0.43	0.33	0
Total 1/2 cys[c]	7.56	8.73	9.34
RAAA [%][d]	88.0	92.8	86.6

[a] This value is a minimum due to partial oxidation to the sulphone.

[b] Methionine Sulphone.

[c] Sum of half cystine and cysteic acid.

[d] Recovery of anhydroamino acids.

TABLE 3.8.

AMINO ACID ANALYSES [MOLE %] OF THE HIGH-SULPHUR [SCMKB] PROTEINS OF WOOL.

Amino Acid	SCMKB Wool	SCMKB Gold-stained Wool	SCMKB Wool. Gillespie and Inglis (1965)
Cysteic acid	0.25	2.31	-
Aspartic acid	4.67	3.50	2.97
Threonine	9.44	9.67	10.25
Serine	13.31	15.19	12.71
Glutamic acid	9.53	6.86	8.43
Proline	10.58	12.24	12.45
Glycine	7.19	8.04	6.88
Alanine	3.62	3.37	2.92
Valine	5.74	5.52	5.64
Half cystine	1.21	1.19	0
Methionine	0.09	0.07	0
Isoleucine	3.15	2.79	3.56
Leucine	5.02	4.12	3.87
Tyrosine	2.48	2.34	2.12
Phenylalanine	2.23	1.87	1.88
Lysine	1.04	0.80	0.61
Histidine	0.78	0.87	0.79
Arginine	5.57	5.56	5.94
SCM-cysteine[a]	14.06	13.63	18.94
Total 1/2 cys[b]	15.52	17.13	18.94
RAAA [%][c]	99.3	97.1	105.8

[a] S-carboxymethylcysteine

[b] Sum of cysteic acid, half cystine and SCM-cysteine

[c] Recovery of anhydroamino acids.

TABLE 3.9.

AMINO ACID ANALYSES [MOLE %] OF THE LOW-SULPHUR [SCMKA] PROTEINS OF WOOL

Amino Acid	SCMKA Wool	SCMKA Gold-stained Wool	SCMKA Wool. Harrap and Gillespie (1963)
Cysteic acid	0.10	0.95	-
Aspartic acid	9.17	9.29	8.31
Threonine	4.80	4.70	4.70
Serine	9.72	9.29	8.94
Glutamic acid	14.47	16.47	13.82
Proline	4.04	3.63	3.88
Glycine	9.04	7.51	9.31
Alanine	6.16	6.53	6.04
Valine	4.95	5.43	5.51
Half cystine	0.28	0.26	Trace
Methionine	0.35	0.43	0.44
Isoleucine	3.01	3.35	3.34
Leucine	9.42	9.98	9.73
Tyrosine	4.52	3.68	4.89
Phenylalanine	3.16	3.43	3.15
Lysine	2.94	3.88	3.43
Histidine	0.74	0.78	0.54
Arginine	6.96	7.11	7.05
SCM-cysteine	6.12	3.23	6.95
Total 1/2 cys[a]	6.41	4.44	6.95
RAAA [%][b]	95.1	85.0	95.3

[a] Sum of cysteic acid, half cystine and SCM-cysteine.

[b] Recovery of anhydroamino acids.

TABLE 3.10.

AMINO ACID ANALYSES [MOLE %] OF THE HIGH-SULPHUR [SCMKB] PROTEINS OF THE ORTHO- AND PARA-CORTICAL CELLS AND GOLD-STAINED WOOL.

Amino Acid	SCMKB Orthocortical Cells	SCMKB Paracortical Cells	SCMKB Gold-stained Wool
Cysteic acid	3.33	3.83	2.31
Aspartic acid	4.43	4.13	3.50
Threonine	9.01	9.33	9.67
Serine	14.03	14.90	15.19
Glutamic acid	8.34	9.95	6.86
Proline	9.82	10.34	12.24
Glycine	10.54	10.05	8.04
Alanine	3.68	3.62	3.37
Valine	5.38	5.07	5.52
Half cystine	1.03	1.82	1.19
Methionine	0.03	0.10	0.07
Isoleucine	2.97	2.78	2.79
Leucine	5.05	4.23	4.12
Tyrosine	3.36	2.68	2.34
Phenylalanine	2.73	2.31	1.87
Lysine	1.04	1.14	0.80
Histidine	0.65	0.93	0.87
Arginine	6.18	5.24	5.56
SCM-cysteine	8.39	7.55	13.63
Total 1/2 cys[a]	12.75	13.20	17.13
RAAA [%][b]	96.3	99.9	97.1

[a] Sum of cysteic acid, half cystine and SCM-cysteine.

[b] Recovery of anhydroamino acids.

TABLE 3.11.

AMINO ACID ANALYSES [MOLE %] OF THE LOW-SULPHUR [SCMKA] PROTEINS OF THE ORTHO- AND PARA-CORTICAL CELLS AND GOLD-STAINED WOOL.

Amino Acid	SCMKA Orthocortical Cells	SCMKA Paracortical Cells	SCMKA Gold-stained Wool
Cysteic acid	1.14	1.19	0.95
Aspartic acid	9.88	10.64	9.29
Threonine	4.68	4.98	4.70
Serine	9.01	8.77	9.29
Glutamic acid	16.81	17.76	16.47
Proline	3.40	2.83	3.63
Glycine	7.76	7.38	7.51
Alanine	6.41	7.00	6.53
Valine	5.50	5.32	5.43
Half cystine	0.33	0.44	0.26
Methionine	0.10	0.17	0.43
Isoleucine	3.35	3.31	3.35
Leucine	10.62	10.18	9.98
Tyrosine	3.69	3.30	3.68
Phenylalanine	3.17	2.82	3.43
Lysine	3.79	3.94	3.88
Histidine	0.65	0.73	0.78
Arginine	6.87	7.03	7.11
SCM-cysteine	2.87	2.17	3.23
Total 1/2 cys[a]	4.34	3.80	4.44
RAAA [%][b]	94.3	94.2	85.0

[a] Sum of cysteic acid, half cystine and SCM-cysteine.

[b] Recovery of anhydroamino acids.

of the gold-stained wool. Tables 3.8 to 3.11 show the amino acid analyses of the high- and low-sulphur proteins from wool, gold-stained wool, orthocortical cells and paracortical cells. For comparison, the amino acid compositions of the high-sulphur proteins from wool obtained by Gillespie and Inglis (1965), and of the low-sulphur proteins from Harrap and Gillespie (1963), are also included.

The recovery of anhydroamino acids from the gold-stained wool, orthocortical cells, paracortical cells and the low-sulphur proteins of these samples, are all low due to the presence of gold. If the percentage of gold [from table 3.3] is added to the percentage recovery of amino acids, then all of the material in the samples is accounted for, within experimental error, apart from the orthocortical cells where only 91.1% is accounted for. All the other recoveries are high and within experimental error.

Comparison of the analyses of wool and gold-stained wool, shows that about 17% of the cystine had been converted to cysteic acid, presumably by the gold chloride being reduced to gold. If the reaction between gold chloride and cystine is

$$3[-CH_2-S-] + 5Au^{+++} + 9H_2O \longrightarrow 3[-CH_2-SO_3H] + 5Au + 15H^+$$

i.e. 3 moles of half cystine reduce 5 moles of gold chloride to gold producing 3 moles of cysteic acid, then the amount of gold reduced by cystine can be calculated from the amount of cysteic acid found in the hydrolysate, assuming that all the cysteic acid results from oxidation of cystine. This is a

valid assumption, as cortical cells isolated by a similar ultrasonic treatment to that used in this investigation, contained only 0.19 mole % [17 micromoles per gm of cortical cells] [Bradbury, Chapman and King (1965a)].

In the gold-stained wool hydrolysate, there was 143 micromoles [per gm of the wool] of cysteic acid. Using the above equation, this is equivalent to 4.69 gm of gold per 100 gm of wool, i.e. the wool contained 4.69% gold. The actual gold content of the wool was found to be 9.43% [from table 3.3]. Thus the amount of cystine oxidized to cysteic acid only accounts for about half [49.7%] of the gold chloride reduced to gold in the fibre. As stated earlier, the remainder of the gold appears to be produced by reduction of the gold chloride by formic acid.

Similar calculations for the orthocortical cells, which contained 236 micromoles of cysteic acid per gm of material, and for the paracortical cells, which contained 228 micromoles of cysteic acid per gm of material, indicates that 7.75 gm and 7.4 gm of gold chloride per 100 gm of cortical cells respectively would be reduced. These values are considerably higher than the actual amounts of gold present, 3.11% and 6.44% [w/w] respectively [from table 3.3]. However, the above calculations are probably not valid, as both cortical cell hydrolysates contain considerably higher cysteic acid contents than the gold-stained wool hydrolysate. Also, methionine sulphone was found present in both of the cortical cell

hydrolysates, but was not present in the gold-stained wool hydrolysate. This indicates that oxidation of methionine, and therefore possibly of cystine, has occurred during isolation, separation, hydrolysis, or analysis of the cortical cells.

Comparison of the gold-stained wool and unstained wool analyses, shows that apart from cysteic acid, cystine and tyrosine, the analyses agree within experimental error. The cysteic acid content is higher and cystine lower in the gold-stained wool, due to oxidation of the cystine by the gold chloride. Comparison of the total half-cystine contents indicates that the gold-stained wool has a slightly lower content [7%] than the unstained wool. The tyrosine content of the gold-stained wool is significantly lower [13%].

On comparing the orthocortical cell analysis with the paracortical cell analysis, it is seen that histidine [13.5%] and total cystine [13.5%] are significantly lower. Tyrosine [26%], glycine [16%], leucine and phenylalanine [both 12%], are all significantly higher in the orthocortex and the total of methionine and methionine sulphone is probably higher, although there is some doubt as to the accuracy of the methionine sulphone values. All the other amino acids agree within experimental error.

The above differences, apart from tyrosine, are all relatively small. Both cortical cell analyses show close agreement with the gold-stained wool, apart from the total cystine contents, which are lower than in the wool. However,

as wool cuticle has a high cystine content [see section 2C(ii)], these values do not appear to be unreal. The average value for the ortho- and para-cortical cells [8.14 mole %] is lower than the value reported by Bradbury, Chapman and King (1965a) for cortical cells isolated by a similar method [9.06 mole %]. The difference is probably just significant. Thus it appears that some cystine may have been destroyed or oxidized e.g. to cystine monoxide, during the staining or isolation procedures.

The difference in dyeing properties of the two cortices has been thought to be due to the orthocortex being acidic [due to an excess of acidic groups] and the paracortex basic [due to an excess of basic groups]. The total of the acidic amino acids [glutamic acid and aspartic acid] is 19.77 mole % for the orthocortex and 20.46 mole % for the paracortex. These are not significantly different. However, the values also include the glutamic acid and aspartic acid formed from their amides during the hydrolysis. No estimate of the amount of each amide in either cortex has been made, and thus the actual difference in total free acidic amino acids is not known. There is no difference in the total of the basic amino acids [lysine, hystidine and arginine] between the two cortices, the orthocortical cells containing 10.61 mole % and the paracortical cells 10.69 mole %. Thus the dyeing phenomenon does not appear to be due to one segment being acidic and the other basic, unless there is a significant difference in acid amide content between the two cortices.

Amino acid analyses of the high-sulphur proteins extracted from the wool and gold-stained wool, show reasonable agreement and also compare well with the analysis reported by Gillespie and Inglis (1965) [see table 3.8]. The only major variation is the total half-cystine content of the wool, which is considerably lower than the other two values. Small but significant amounts of methionine were found to be present in all the high-sulphur protein hydrolysates. Gillespie and Inglis (1965) state that methionine is not present in hydrolysates of high-sulphur proteins.

If a comparison of other analyses published for high-sulphur proteins from normal Merino wools is made [e.g. in Gillespie and Inglis (1965), Gillespie (1964) and Gillespie, Reis and Schinckel (1964)], then it can be seen that large variations occur. Thus the agreement between the results for wool and those of Gillespie and Inglis (1965) for Merino 64's wool, isolated by basically the same method, is acceptable.

The same is true for the low-sulphur proteins from the wool and gold-stained wool [see table 3.9]. The differences between these and the analysis reported by Harrap and Gillespie (1963), show no greater variation than between those reported by Harrap and Gillespie (1963), Gillespie (1964) and Gillespie, Reis and Schinckel (1964) for normal Merino wools.

Comparing the high-sulphur analyses of the ortho- and para-cortical cells, it can be seen again that there are very few differences. Methionine is present, although in very small

amounts. Glutamic acid, histidine and total half cystine are lower, and leucine and tyrosine higher in the orthocortical cells than in the paracortical cells. The most striking feature of the analyses, is the close agreement with the high-sulphur protein analyses of the gold-stained wool and wool, apart from the lower total cystine contents. This is in keeping with the low cystine contents of the ortho- and para-cortical cells. A fraction rich in cystine from the cuticle may account for the difference.

The low-sulphur proteins from the ortho- and para-cortical cells are also the same within experimental error, apart from proline which is higher in the orthocortical cells. The methionine values are low, and since methionine sulphone was observed in the hydrolysates of the cortical cells, this is probably the reason. If it is, then the SCM-cysteine values will be high, as SCM-cysteine and methionine sulphone are eluted from the ion-exchange column together. The error in the SCM-cysteine values will only be small [less than 10%], as there is very little methionine in the samples.

The differences in composition between the two cortices derived from the values that have been reported in the literature, are summarized in table 3.12. In table 3.13, a brief outline of the methods used to prepare the samples for the analyses in table 3.12 is given. The differences in composition between the two cortices found in this investigation are also included for comparison. The symbols that are used and their meaning are as

TABLE 3.12.

SIGNIFICANT DIFFERENCES IN COMPOSITION OF THE ORTHOCORTEX WHEN COMPARED TO THE PARACORTEX.

Amino Acid	Golden et al. (1955)	Leveau (1959e)	Schoberl (1960)	Haly et al. (1964)	Leach et al. (1964)	Miro et al. (1965)	Horio et al. (1965)	Present Work
Asp	H	-	-	H	-	-	H	-
Thr	H	H	L	-	-	L	H	-
Ser	-	-	-	L	-	-	H	-
Glu	H	-	-	-	H	-	-	-
Pro	H	L	L	-	L	-	-	-
Gly	H	-	H	-	H	H	-	H
Ala	-	-	-	-	H	-	-	-
Val	H	-	-	H	-	-	-	-
Cys	L	L	L	L	L	L	L	L
Met	O	O	O	-	H	L	-	H
Ileu	O	-	-	H	-	-	-	-
Leu	-	H	-	-	H	-	-	H
Tyr	O	H	-	L	H	-	-	H
Phe	-	H	H	-	H	H	-	H
Lys	O	-	-	-	H	H	L	-
His	O	-	O	L	L	H	H	L
Arg	L	L	O	L	H	H	-	-

L Lower in the orthocortex compared to the paracortex.
H Higher in the orthocortex compared to the paracortex.
- No significant difference.
O No values reported.

TABLE 3.13.

METHODS USED TO ISOLATE THE ORTHO- AND PARA-CORTICES FOR THE ANALYSES IN TABLE 3.12

Author(s)	Method
Golden, Whitwell and Mercer (1955)	Wool treated with water, 130°C, 90 min; orthocortex digested by trypsin.
Leveau (1959e)	Wool partially hydrolysed with 6N HCl, 40°C, 6 hr; two cortices mechanically separated.
Schoberl (1960)	Similar method to Leveau (1959e).
Haly and Inglis (1964)	Lincoln wool fibres abraded until only central orthocortex remained.
Leach, Rogers and Filshie (1964)	Dissolved orthocortex in HCl, pH 2, 100°C, 96 hr.
Miro and Blade (1965)	Same method as Leveau (1959e).
Horio et al. (1965)	Wool heated in water, 170°C, 6 hr; two cortices separated on density gradients.
Present work	Wool bilaterally stained with gold; fibres dispersed by ultrasonics; cortical cells separated on density gradients.

follows: '-' indicates no significant difference for the amino acid; 'H' and 'L' indicate that the value for the amino acid is significantly higher or lower [respectively] in the orthocortex compared to the paracortex; and '0' indicates that no value was reported for the amino acid.

It can be seen that the only consistent difference, in all the analyses, is the lower content of cystine in the orthocortical cells compared to the paracortical cells. The earlier results indicated that the difference in cystine content between the two cortices was of the order of 100% to 200%, but it is in fact only 13% to 14% lower in the orthocortical cells. There is no other consistency throughout the table.

The results of histochemical analyses, as well as results from staining and other chemical observations for individual amino acids, are shown in table 3.14. The means of analyses are also summarized.

Of the amino acid analyses that have been previously reported, [see table 3.12], none can be regarded as giving the unequivocal composition of the "native" ortho- and para-cortices.

Golden, Whitwell and Mercer (1955) analysed a sample of paracortex contaminated with epicuticle, and the amino acid composition of the orthocortex that they report was calculated from the paracortex composition. Also the analysis was incomplete. The analysis performed by Schoberl (1960), is incomplete and the author does not state the units for the values quoted. Haly and Inglis (1964) only analysed the orthocortex [of Lincoln wool],

TABLE 3.14.

RESULTS OF ANALYSES NOT RECORDED IN TABLE 3.12 FOR AMINO ACIDS WHICH ARE HIGHER [H], LOWER [L] OR EQUAL [=] IN THE ORTHOCORTEX WHEN COMPARED TO THE PARACORTEX.

Amino Acid	Result and Reference
Aspartic acid	H [2]
Threonine	= [4]
Serine	H [4]
Glutamic acid	H [2]: L [4]
Proline	L [2]
Glycine	= [4]
Cystine	L [2,3,4, 7,9,14,16]: = [5,6,8,17]
Tyrosine	H [1,15]: = [10]
Arginine	L [4]: = [5,10]
Tryptophan	= [12]
Total basic amino acids	L [11,13,18]
Total acidic amino acids	H [11,13,18]

REFERENCES FOR TABLE 3.14.

[1] Mercer (1954e); Nickel staining studies.

[2] Lindley (1947), from Fraser, Lindley and Rogers (1954): Partial hydrolysis with cetylsulphonic acid, 0.05M, 65°C, 6 days - orthocortex dissolved in NaOH.

[3] Mercer, Golden and Jeffries (1954): Cystine analyses on paracortex - orthocortex dissolved by enzymes.

[4] Derminot and Leveau (1956): Two cortices separated after partial acid hydrolysis - analysis by paper chromatography and electrophoresis. [Refs. contd. next page]

[5] Derminot (1958): Two cortices separated after partial acid hydrolysis - analysis by "chromatoelectrophoresis".

[6] Ryder (1956, 1958): Observations of incorporation of radioactive ^{35}S in the growing fibre.

[7] Leveau (1957): Analyses of the two cortices separated after partial acid hydrolysis of the fibres.

[8] Leveau (1958): As for 7 - regards results in 7 wrong.

[9] Menkart and Coe (1958): Staining with mercury vapour.

[10] Ryder (1959): Histochemical stains - mild treatment indicated a difference in the cortices; vigorous treatment showed equal distribution of the amino acids.

[11] Horio et al. (1960): Dyeing with acid and basic dyes.

[12] Miro (1961): Analyses of the two cortices separated after partial acid hydrolysis of the fibres.

[13] Williams (1962): Results from staining wool with ninhydrin.

[14] Haly (1963): From supercontraction data, he estimated that the cystine content of the orthocortex to be 0.8 to 0.95 times that in the paracortex, of Corriedale wool.

[15] Corbett and Yu (1964): Staining with metal ions.

[16] Kassenbeck (1965a,b): Results of staining wool with mercury and silver salts.

[17] Parisot, Allard and Baures (1965): Analyses of the two cortices separated after partial acid hydrolysis of the fibres.

[18] Appleyard and Lees (1965): Results of staining wool with fluorescent dyes.

and due to the small sample size, is only of moderate accuracy. Leach, Rogers and Filshie (1964) analysed impure samples of both cortices; the "paracortex" sample contained exocuticle, while the orthocortex contained endocuticle.

It is known that some peptide bonds are more stable than others to acid hydrolysis [e.g. see Hill (1965)]. It seems unlikely that no splitting of peptide bonds would occur in the paracortex during even the mildest acid treatment used by any of the above authors. If peptide bonds are broken in the paracortex, then it is inevitable that short polypeptides will be formed that can diffuse from the protein. These only have to be slightly enriched in a few amino acids to give a false idea of the amino acid composition of the "native" protein.

That small peptides rich in a few amino acids are formed and diffuse from the fibre during the short partial acid hydrolysis used by Leveau (1959e) and Miro and Blade (1965), can be seen in the analysis of the wool after the partial hydrolysis when compared to the analysis of the untreated wool. Thus the results obtained by Leveau (1959e), Schoberl (1960), Leach, Rogers and Filshie (1964) and Miro and Blade (1965), do not indicate the true composition of the unmodified "native" proteins.

The protein in the two cortices, separated after heating wool at 170°C in water [Horio et al. (1965)], is considerably degraded, as evidenced by the low cystine content especially, and low threonine and serine contents of the wool after the

treatment. This also applies to the wool heated at 130°C [see Horio et al. (1965)], which must imply that the supercontracted wool used by Mercer, Golden and Jeffries (1955) was modified even before the trypsin further attacked the protein.

Leveau (1959e) after considering his results and the results of others, concluded that the values obtained closely followed the method of preparation of the two cortices. He also concluded that the ortho- and para-cortices in the "native" keratin probably had very similar compositions.

There has been considerable attention devoted to the amount of cystine in the two cortices [see table 3.14]. Originally, it was thought that the paracortex was more chemically stable, due to the presence of more disulphide cross links and therefore more cystine, than the orthocortex. Ryder (1956, 1958), using ^{35}S labelled cystine, was the first to suggest that the difference in cystine content between the two cortices was small. Later, Leveau (1958) agreed and suggested that the different reactivities of the two cortices was due to different incorporation of cystine into the polypeptide chains; the orthocortex containing intrachenic bound cystine and the paracortex interchenic bound cystine in the polypeptide chains. However, Kassenbeck (1965b) not only claims that there is more than twice the cystine content in the paracortex, as compared to the orthocortex, but that the cystine in the paracortex will be bound intrachenically and in the orthocortex by interchenic bonds. This is the converse to Leveau's suggestion.

Haly (1963), studying supercontraction phenomena on Corriedale wool, postulated that the cystine content of the orthocortex was about 0.85 to 0.9 that of the paracortex.

Simmonds and Bartulovich (1958) analysed "light" and "heavy" cortical cells from wool. It was implied that the "heavy" cells were derived from the paracortex and the "light" cells from the orthocortex [Ward and Bartulovich (1955)]. However, this has never been definitely proved and thus the analyses can not be taken as indicating the composition of the two cortices. The cortical cells were obtained from wool after a partial acid hydrolysis, and thus would also be chemically degraded.

[E] CONCLUSIONS.

As pointed out earlier, Bradbury, Chapman and King (1965a) have shown that the method used to obtain cortical cells [ultrasonic disintegration of the wool fibres in formic acid], causes no apparent chemical modification of the wool, cortical cells or cuticle. There is some material dissolved by the formic acid during the treatment, but this is mainly cell membrane and nuclear remnant material [Bradbury, Chapman and King (1965b)]. All the previous methods used to separate the two cortices for the analyses shown in table 3.12 [apart from the method of Haly and Inglis (1964)], cause chemical modification of the protein and therefore must be treated with reserve. Although the method that Haly and Inglis (1964) used was not chemically degradative, they could only obtain orthocortical material for analysis and compare this with the whole fibre composition. Thus this is the first analysis of ortho- and para-cortical cells in which there has been no detectable chemical modification.

That the two cortices have similar amino acid compositions and contents of high- and low-sulphur proteins of almost the same composition, was a surprising result.

From table 3.7 , it will be seen that the ratio of [total] cystine in the two cortical cells is 1:0.87. This agrees remarkably well with the ratio predicted by Haly (1963) for

Corriedale wool from supercontraction data; i.e. the cystine content of the orthocortex would be between 0.8 and 0.95 that of the paracortex. The lower cystine content in the orthocortical cells appears to be due to a lower cystine content in its high-sulphur proteins, rather than due to a difference in the amounts of high- and low-sulphur proteins in the two cortices.

Do the findings from this investigation shed any light on the nature of the differences in the ortho- and para-cortex? They discount the theory that the bilateral dyeing is due to a different amino acid composition of the two cortices. However, amino acid analyses do not give any information as to the sequence of the amino acids in the polypeptide chains or the folding of the chains. There does not appear to be a difference in the acidity [or basicity] of the two cortices, unless there is a considerable difference in amide content, to account for the differing dye uptake of the two cortices. To dye molecules, the orthocortex could appear to be acidic if the acidic groups are free and the basic groups are buried in hydrophobic areas of the protein. Conversely, the paracortex could appear to be basic due to the acidic groups being buried in hydrophobic areas.

This does not appear to be a reasonable explanation, as all the ionic groups in wool can be titrated and are thus accessible [Alexander, Hudson and Earland (1963)]. It has also been shown in several soluble proteins, that none of the polar

side-chains [i.e. the side-chains of acidic and basic amino acids] are buried in hydrophobic regions of the molecules. In horse oxyhaemoglobin, Perutz (1965) has shown that all the polar side-chains are on the surface of the molecule, and the non-polar side-chains are buried in the hydrophobic regions. A similar result was found for lysozyme [Blake et al. (1967)]. Thus it would not appear that acidic or basic groups would be buried in hydrophobic regions in the wool.

There can be no doubt that the different morphological structure of the two cortices plays a large part in determining the differences between the cortices. The cell membranes have no effect on the different dyeing properties of the two cortices, as removal of them by formic acid does not affect the differential dye uptake [see section 3A]. The cuticle has no effect either, as Mercer (1954a) showed that after removal of the cuticle, bilateral staining could still be achieved.

Leach, Rogers and Filshie (1964) have put forward two possible explanations for the observed differences in chemical reactivity of the two cortices. One is an extrapolation from the known different histological structure in the two cortices, to include differences at the molecular level; i.e. individual polypeptide chains more closely packed and therefore more resistant to chemical attack in the paracortex than in the orthocortex. The other is based on the different ratio of matrix to filaments in the two cortices.

The difference in cystine content of the two cortices was

originally thought to give rise to the differing reactivity of them, the paracortex having a higher number of disulphide cross links than the orthocortex. The small difference in cystine content would not be enough to account for the observed different chemical stability of the two cortices, unless there was a different ratio of inter- and intra-chenic disulphide bonds, as was originally suggested by Leveau (1958).

The above, together with the first hypothesis of Leach, Rogers and Filshie (1964), appears to be the most likely explanation. That is, the individual polypeptide chains are more closely packed in the paracortex than in the orthocortex, together with a possible difference in the ratio of inter- to intra-chenic disulphide bonds between the two cortices.

4. APPENDICES.

[A] SOME INTERESTING OBSERVATIONS RELATED TO THE ORTHO- AND PARA-CORTEX.

Dinitrophenylated [DNP'd] wool, which is yellow in colour, was partially bleached when treated with chlorine water. The bleaching action appeared to be associated with the asymmetry of the cortex, the inside of the crimp wave always being the segment which lost its colour and became white. This possibly indicates that the paracortex is the segment which is bleached. An overtreatment with saturated chlorine water was found to bleach the other segment also. The yellow colour could be returned by immersion in ethanol for 12 hours, water for 3 days, or dilute sodium hydroxide or sodium hydrosulphite for 5 minutes [before the fibres dissolved].

Dispersion of the "white-yellow" fibres, by treatment with ultrasonics [in formic acid] using the method of Bradbury and Chapman (1964), gave a suspension of yellow cortical cells only - no white cortical cells could be observed, although the fibres were still bilaterally "coloured" after the treatment.

It was found that after a chlorine water treatment [with saturated chlorine water, diluted 10:1 with water, for 1 hour], the DNP'd wool initially dissolved rapidly in formic acid, about 45% being dissolved in less than 24 hours. After 24 hours, the wool dissolved at a slower rate, as shown in figure 4.1. Untreated wool also followed a similar dissolution curve in formic acid, after treatment with chlorine water [see figure 4.1].

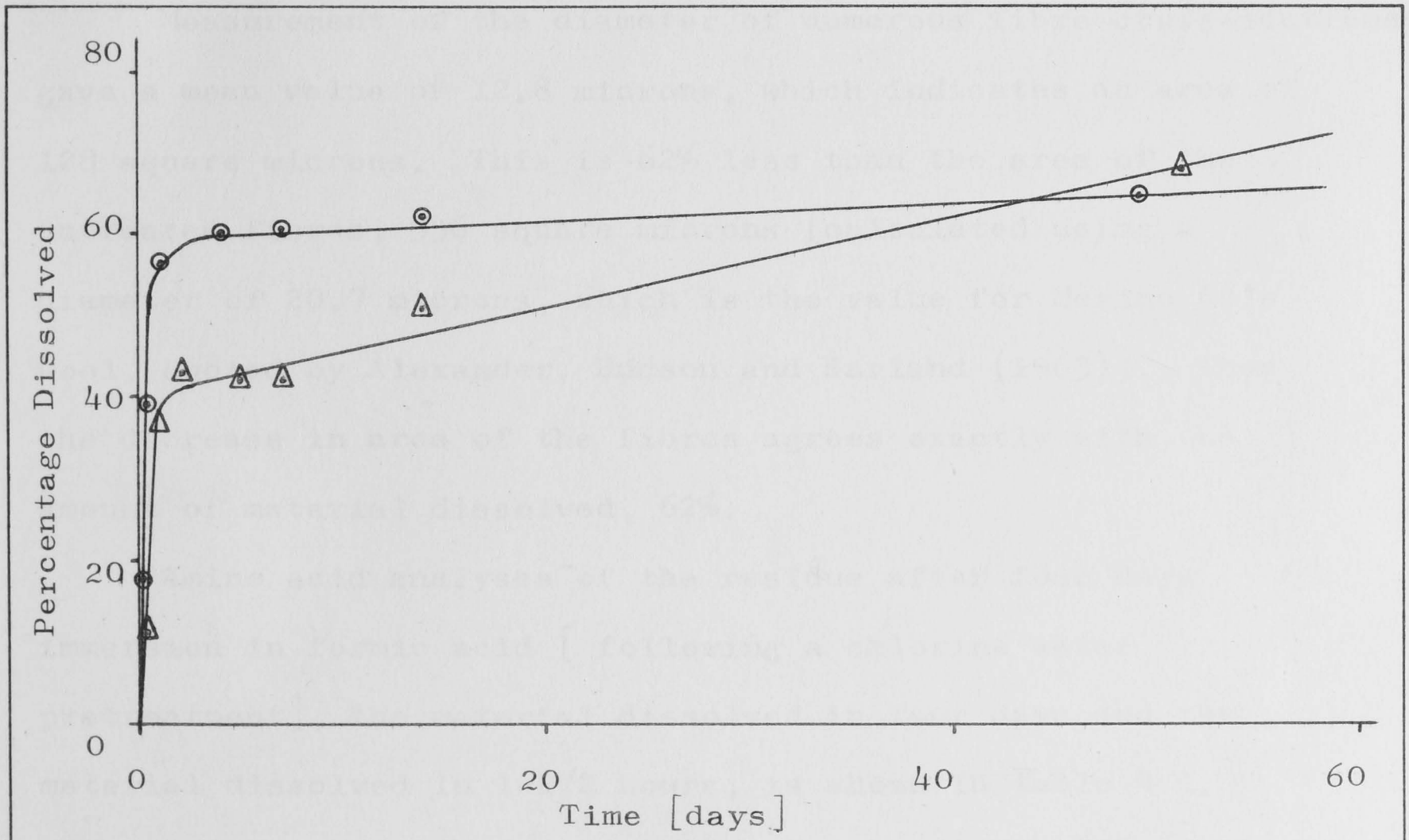


Figure 4.1. Material dissolved from wool [⊙] and DNP'd wool [△] [pretreated with chlorine water] in formic acid.

The amount dissolved from wool, levelled out at about 62%. This was thought to indicate that the orthocortex had dissolved, as there is about 60% orthocortex in the fibre [see section 1B], leaving behind the paracortex. Cross-sections of the fibres remaining after four days immersion in formic acid, showed that they were still round with no sign of one segment missing. Figure 4.2 is an electron micrograph of a section at low magnification [stained by the TGA-OsO_4 procedure of Rogers (1959b)] and is shown at higher magnification in figure 4.3.

From figure 4.2, it can be seen that all of the cuticle has been dissolved. In figure 4.3, the remaining fibrils and matrix appear to be quite normal in appearance, indicating that no morphological component is preferentially dissolved.

Measurement of the diameter of numerous fibre cross-sections, gave a mean value of 12.8 microns, which indicates an area of 128 square microns. This is 62% less than the area of the untreated fibres, 336 square microns [calculated using a diameter of 20.7 microns, which is the value for Merino 64's wool, quoted by Alexander, Hudson and Earland (1963)]. Thus the decrease in area of the fibres agrees exactly with the amount of material dissolved, 62%.

Amino acid analyses of the residue after four days immersion in formic acid [following a chlorine water pretreatment], the material dissolved in four days and the material dissolved in 1-1/2 hours, is shown in table 4.1.

The residue remaining after four days [i.e. 40% of the

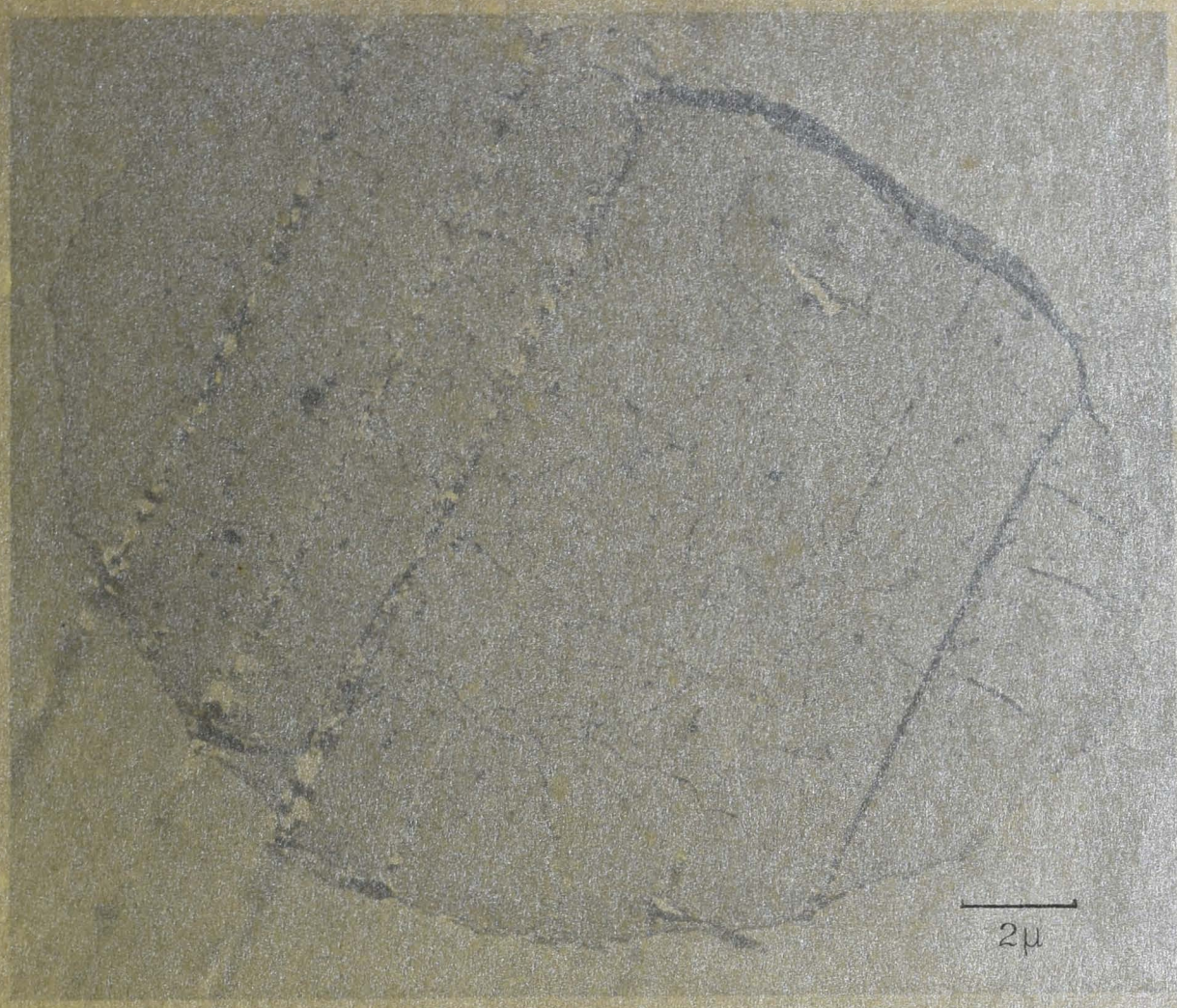


Figure 4.1. Electron micrograph of a cross-section of a wool fibre treated with chlorine water and formic acid.

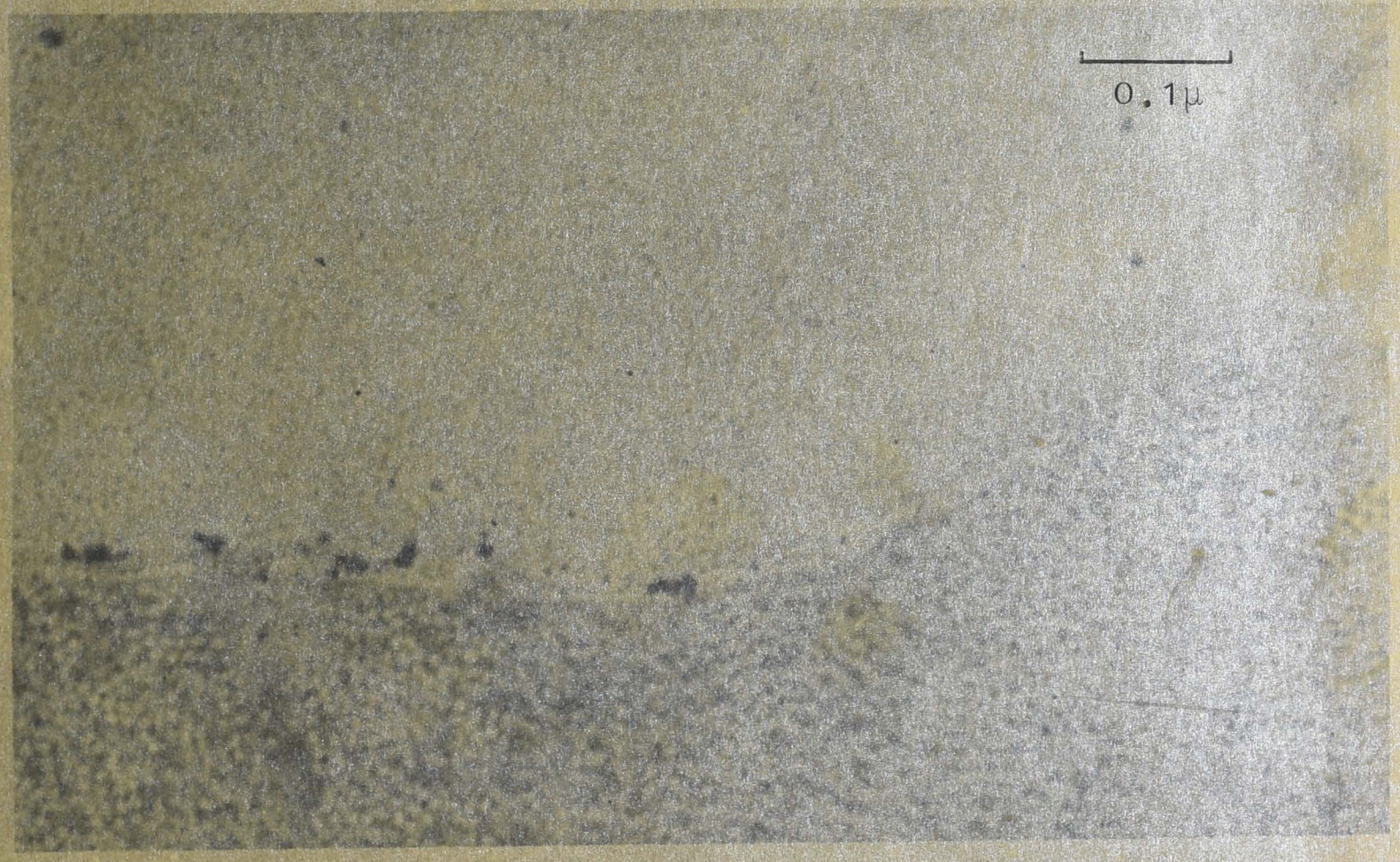


Figure 4.2. High magnification electron micrograph of part of the wool fibre in Figure 4.1.

TABLE 4.1.

AMINO ACID ANALYSES [MOLE %] OF THE RESIDUE AND DISSOLVED MATERIAL FROM WOOL[a] AFTER 4 DAYS IMMERSION IN FORMIC ACID, AND MATERIAL DISSOLVED DURING 90 MINUTES IMMERSION.

Amino Acid	Residue [4 days]	Dissolved Material		Wool[b]
		4 days	90 min	
$CySO_3H$	3.90	5.16	4.91	0.06
Asp	6.70	8.39	8.31	6.52
Thr	6.22	5.85	5.83	6.63
Ser	10.45	10.58	10.25	10.69
Glu	13.14	13.77	14.44	12.18
Pro	6.48	5.90	6.04	6.77
Gly	8.56	9.03	7.61	8.33
Ala	5.47	6.02	6.07	5.33
Val	5.29	6.05	6.56	5.51
1/2 Cys	6.22	0	0	9.99
Met	0.35	0	0	0.38
Ileu	3.04	3.50	3.66	3.08
Leu	7.44	8.87	9.54	7.61
Tyr	2.81	0.08	0.08	3.89
Phe	2.82	3.27	3.24	2.72
Lys	3.21	3.47	3.29	2.97
His	0.78	0.39	0.33	0.87
Arg	6.58	7.08	7.37	6.48
Cl Tyr[c]	0.44	0.71	0.18	-
DiCl Tyr[d]	0.07	1.86	2.28	-
Total 1/2 Cys	10.12	5.16	4.91	10.05
Total Tyr	3.32	2.65	2.54	3.89
RAAA [%][e]	80.9	81.7	75.7	97.0

[a] Wool given a pretreatment in chlorine water for 1 hour.

[b] Untreated wool: From table 3.6.

[c] Monochlorotyrosine.

[d] Dichlorotyrosine: Values are approximate.

[e] Recovery of anhydroamino acids.

original fibre] has the same composition as untreated wool [within experimental error], except for a slightly lower tyrosine content [15% lower]. The total cystine content is the same, although 26% of the cystine has been converted to cysteic acid. The analyses of the material dissolved during four days immersion and 1-1/2 hours immersion are the same within experimental error, apart from glycine shich shows a 16% difference. The only amino acids which show significant differences when compared with the residue or wool analyses are aspartic acid, glutamic acid, isoleucine, leucine, phenylalanine, histidine, total cystine and total tyrosine.

Thus, material with almost the same composition as wool, has been dissolved leaving a residue [40% of the original wool] with unaltered composition. Apparently the wool is being evenly dissolved from the outside of the fibres.

[B] AMINO ACID ANALYSES OF SOME OTHER KERATIN FIBRES.

Observation of Seal hair under the microscope, showed that it consisted of two types of fibres. One type consisted of very fine fibres about 20 - 30 microns in diameter, and the other, coarse flat fibres having an oval cross-section of major diameter about 160 microns and minor diameter about 30 microns. Only the coarse fibres were medullated.

The fine fibres were separated from the coarse fibres by hand picking, using a stereomicroscope. The results of amino acid analyses of the two types of fibres are shown in table 4.2. For comparison, an analysis of Seal hair by Menkart (1966) is included. The reason for the low yields is unknown.

There is very little difference to be found between all three analyses. Citrulline was found in the hydrolysates of both the fine and coarse fibres, the fine fibres containing twice that in the coarse fibres. Menkart has no figure for citrulline or ornithine. Why the citrulline content of the unmedullated fibres is twice that of the medullated fibres, is not known. The glycine content of the fine fibres is slightly lower than in the other samples. Alanine is slightly low and tyrosine high in the coarse fibre. The cystine values show the biggest differences. The higher cysteic acid content of the coarse "guard hair" fibres, is probably due to these fibres being more weathered than the fine "underhair" fibres. The

TABLE 4.2.

AMINO ACID ANALYSES [MOLE%] OF SEAL HAIR.

Amino Acid	Fine Seal Hair	Coarse Seal Hair	Seal Hair Menkart (1966)
Cysteic Acid	0.16	0.25	-
Aspartic Acid	5.46	5.24	5.61
Threonine	6.34	6.00	6.58
Serine	11.33	10.47	9.64
Glutamic Acid	10.13	9.70	10.39
Proline	7.42	6.97	7.72
Citrulline	0.13	0.06	-
Glycine	10.18	11.78	11.54
Alanine	5.02	4.66	6.27
Valine	4.86	4.50	4.35
1/2 Cystine	14.88	16.13	12.75
Methionine	0.92	0.86	1.09
Isoleucine	2.59	2.51	2.44
Leucine	6.08	5.78	6.18
Tyrosine	3.37	4.04	3.63
Phenylalanine	2.03	1.91	2.36
Ornithine	0	0	-
Lysine	2.25	2.17	2.10
Histidine	0.70	0.76	0.63
Arginine	6.13	6.23	6.71
RAAA [%][a]	65.3	85.6	93.0

[a] Recovery of anhydroamino acids.

TABLE 4.3.

AMINO ACID ANALYSES [MOLE %] OF MUTANT MERINO WOOL, MERINO WOOL[a], SWANS DOWN AND GOOSE DOWN [SCHROEDER et al.(1955)].

Amino Acid	Mutant Merino Wool	Merino Wool[a]	Swans Down	Goose Down[b]
$CySO_3H$	0.08	0.06	0.25	-
Asp	6.49	6.52	5.54	6.18
Thr	6.14	6.63	5.00	5.26
Ser	10.43	10.69	14.63	13.91
Glu	12.76	12.18	8.29	6.88
Pro	6.49	6.77	11.41	9.92
Cit	0.11	0.045[c]	0.11	-
Gly	8.23	8.33	11.03	12.47
Ala	5.30	5.33	5.43	5.47
Val	5.55	5.51	7.68	7.80
1/2 Cys	10.02	9.99	7.15	10.86
Met	0.56	0.38	0.28	0.24
Ileu	3.17	3.08	4.79	4.13
Leu	7.50	7.61	7.25	6.71
Tyr	3.77	3.89	1.49	2.22
Phe	2.71	2.72	3.51	2.53
Orn	0.10	0.028[c]	0	-
Lys	2.73	2.97	1.04	1.08
His	0.84	0.87	0.28	0.23
Arg	6.99	6.48	4.90	4.10
Total Cit	0.21	0.073[c]	0.11	-
RAAA [%][d]	91.5	97.0	90.4	95.2

[a] From table 3.6.
[b] From Schroeder et al. (1955).
[c] From King (1967).
[d] Recovery of anhydroamino acids.

larger tyrosine content in the coarse hair compared to the fine hair, is probably due to the higher amount of pigment present [as observed under the microscope], since the pigmented Alpaca fibres were found to have higher tyrosine content than the unpigmented Alpaca fibres [see section 2C (iii)]. The analyses therefore show that there is very little difference in composition of the outer guard hairs and the fine underhair fibres of the Seal.

The analyses of mutant Merino wool shown in table 4.3, is the same as that for normal Merino wool within experimental error, except for the higher methionine [47%] and citrulline [190%] content. The mutant Merino wool fibres are not medullated and thus the high citrulline content is not due to this. It must indicate that the cuticle of the mutant Merino wool has a very high citrulline content. This cannot be confirmed until the cuticle is analysed.

The composition of Swans down is compared with that of Goose down [analysed by Schroeder et al. (1955)] in table 4.3. It can be seen that citrulline is present in these keratins also. The composition of the two downs from the different birds is very similar, with major differences in only six amino acids, glutamic acid, proline, cystine, isoleucine, tyrosine and phenylalanine.

PUBLICATIONS

Publications that are partly or wholly a result of this work are listed below.

1. Separation of Chemically Unmodified Histological Components of Keratin Fibres and Analyses of Cuticles, by Bradbury, J.H., Chapman, G. V., Hambly, A. N. and King, N. L. R. (1966), Nature 210:1333 .

2. The Chemical Composition of Wool VI. Separation and Analyses of the Ortho- and Para-cortex, by Bradbury, J. H. and Chapman, G. V., (1967), Nature. In preparation.

REFERENCES

Ahmad, N. and Lang, W.R. (1956). Text. Res. J. 26:954.

Ahmad, N. and Lang, W.R. (1957). Aust. J. Biol. Sci. 10:118.

Alexander, P. and Earland, C. (1950). Nature 166:396.

Alexander, P., Hudson, R.F. and Earland, C. (1963). "Wool, Its Chemistry and Physics", [Second edition, Chapman and Hall, London].

von Allworden, K. (1916). Z. angew. Chem. 29:77.

Andrews, M.W., Feughelman, M. and Mitchell, T.W. (1962). Text. Res. J. 32:421.

Andrews, M.W., Inglis, A.S. and Williams, V.A. (1966). Text. Res. J. 36:407.

Anon. (1947). "The Tippy Dyeing of Wool and its Control", Amer. Dyestuff Reptr. 36:486.

Appleyard, H.M. and Greville, C.M. (1950). Nature 166:1031.

Appleyard, H.M. and Lees, K. (1965). J. Text. Inst. 56:T38.

Appleyard, H.M. and Perkin, M.E.A. (1963). Personal communication to Ryder (1963).

Asquith, R.S. and Parkinson, D.C. (1966). Text. Res.J. 36:1064.

Auber, L. (1952). Trans. Roy. Soc. Edin. 62:191.

Auber, L. and Ryder, M.L. (1955). Proc. Int. Wool Text. Res. Conf., Aust., 1955 F:36.

Bartulovich, J.J. (1964). Text. Res. J. 34:461.

Baird, K. (1962). Text. Res. J. 32:419.

Baird, K. (1963). Text. Res. J. 33:866.

Bendit, E.G. (1966). Nature 211:1257.

Bendit, E.G. and Feughelman, M. (1967). Quoted in Watt and Morris (1967).

von Bergen, W. (1929). "Wollkunde", [J. Springer, Berlin].

von Bergen, W. (1935). Proc. Amer. Soc. Test. Mater. 35, part II:705.

Birbeck, M.S.C. and Barnicot, N.A. (1959). In "Pigment Cell Biology", [Ed., M. Gordon], P.549, [Academic Press, New York].

Blackburn, S. (1948). Biochem. J. 43:114.

Blake, C.C.F., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. (1967). Proc. Roy. Soc. 167B:365.

Blakey, P.R., Happey, F., Johnson, A.G. and Stell, J.G.P. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:273.

Blout, E.R. (1962). In "Polyamino Acids, Polypeptides, and Proteins", [Ed., M.A. Stahmann], P.275, [University of Wisconsin Press].

Blout, E.R., de Loze, C., Bloom, S.M. and Fasman, G.D. (1960). J. Amer. Chem. Soc. 82:3787.

Bradbury, J.H. (1960). Text. Res. J. 30:128.

Bradbury, J.H. and Chapman, G.V. (1964). Aust. J. Biol. Sci. 17:960.

Bradbury, J.H., Chapman G.V., Hambly, A.N. and King, N.L.R. (1966). Nature 210:1333.

Bradbury, J.H., Chapman, G.V. and King, N.L.R. (1965a). Aust. J. Biol. Sci. 18:353.

Bradbury, J.H., Chapman, G.V. and King, N.L.R. (1965b). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:359.

Bradbury, J.H. and King, N.L.R. (1967). Aust. J. Chem. In press.

Chapman, R.E. (1964). Wool Technol. Sheep Breeding 11:19.

Chapman, R.E. (1965). Aust. J. Biol. Sci. 18:689.

Chapman, R.E. (1965a). In "Biology of the Skin and Hair Growth", [Eds., A.G. Lyne and B.F. Short], P.201, [Angus and Robertson, Sydney].

Clarke, W.H. and Maddocks, I.G. (1965). Stain Technol. 40:339.

Corbett, M.J. and Yu, T.M. (1964). Text. Res. J. 34:655.

Crewther, W.G., Fraser, R.D.B., Lennox, F.G. and Lindley, H. (1965). Advan. Protein Chem. 20:191.

Crewther, W.G., Gillespie, J.M., Harrap, B.S. and Inglis, A.S. (1966). Biopolymers 4:905.

Daveloose, Cl., Mazingue, G. and Van Overbeke, M. (1960). Bull. Inst. Text. France 88:61.

Davies, J.L. (1963a). Text. Res. J. 33:400.

Davies, J.L. (1963b). Text. Res. J. 33:1028.

Davies, J.L. (1965). Text. Res. J. 35:194.

Derminot, J. (1958). Bull. Inst. Text. France 72:69.

Derminot, J. and Leveau, M. (1956). Bull. Inst. Text. France 64:7.

Derminot, J., Tasdhomme, M. and Parisot, A. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:379.

Dobb, M.G. (1964). J. Mol. Biol. 10:156.

Dobb, M.G. (1965). Nature 207:293.

Dobozy, O.K. (1958). Text. Res. J. 28:717.

Dobozy, O.K. (1959). Text. Res. J. 29:836.

Dreywood, R. (1946). Ind. Eng. Chem., Anal. Ed. 18:499.

Dusenbury, J.H. (1960). J. Text. Inst. 51:T756.

Dusenbury, J.H. and Coe, A.B. (1955). Text. Res. J. 25:354.

Dusenbury, J.H. and Jeffries, E.B. (1955). J. Soc. Cosmetic Chem. 6:355.

Dusenbury, J.H. and Menkart, J. (1955). Proc. Int. Wool Text. Res. Conf., Aust., 1955 F:142.

Dusenbury, J.H., Mercer, E.H. and Wakelin, J.H. (1954). Text. Res. J. 24:890.

Einsele, W. (1937). J. Genet. 34:1.

Elliott, R.L., Asquith, R.S. and Rawson, D.H. (1958a). J. Soc. Dyers Colourists 74:173.

Elliott, R.L., Asquith, R.S. and Rawson, D.H. (1958b). J. Soc. Dyers Colourists 74:176.

Elliott, R.L., Asquith, R.S. and Rawson, D.H. (1959). J. Soc. Dyers Colourists 75:455.

Elliott, R.L. and Roberts, J.B. (1956). J. Soc. Dyers Colourists 72:370.

Elliott, R.L. and Roberts, J.B. (1957). J. Soc. Dyers Colourists 73:95.

Feughelman, M. and Haly, A.R. (1960). Text. Res. J. 30:897.

Filshie, B.K. (1967). Private communication.

Filshie, B.K. and Rogers, G.E. (1961). J. Mol. Biol. 3:784.

Fitzpatrick, T.B., Brunet, P. and Kukita, A. (1958). In "The Biology of Hair Growth", [Eds., W. Montagna and R.A. Ellis], p.255, [Academic Press, New York].

Fraser, I.E.B. (1964). Aust. J. Biol. Sci. 17:521.

Fraser, R.D.B., Lindley, H. and Rogers, G.E. (1954). Biochim. Biophys. Acta 13:295.

Fraser, R.D.B. and MacRae, T.P. (1956). Text. Res. J. 26:618.

Fraser, R.D.B. and Rogers, G.E. (1953). Biochim. Biophys. Acta 12:484.

Fraser, R.D.B. and Rogers, G.E. (1954). Biochim. Biophys. Acta 13:297.

Fraser, R.D.B. and Rogers, G.E. (1955a). Aust. J. Biol. Sci. 8:288.

Fraser, R.D.B. and Rogers, G.E. (1955b). Proc. Int. Wool Text. Res. Conf., Aust., 1955 F:106.

Fraser, R.D.B. and Rogers, G.E. (1955c). Proc. Int. Wool Text. Res. Conf., Aust., 1955 F:151.

Fraser, R.D.B. and Rogers, G.E. (1955d). Biochim. Biophys. Acta 16:307.

Freney, M.R. (1947). Nature 160:799.

Garside, J.E. and Phillips, R.F. (1953). "A Textbook of Pure and Applied Chemistry", p.482, [Sir Isaac Pitman and Sons, London].

Geiger, W.B. (1944a). J. Res. Nat. Bur. Stand. 32:127.

Geiger, W.B. (1944b). Amer. Dyestuff Reptr. 33:117.

Gillespie, J.M. (1958). Biochim. Biophys. Acta 27:225.

Gillespie, J.M. (1959). Nature 183:322.

Gillespie, J.M. (1960). Aust. J. Biol. Sci. 13:81.

Gillespie, J.M. (1962). Aust. J. Biol. Sci. 15:572.

Gillespie, J.M. (1963a). Aust. J. Biol. Sci. 16:241.

Gillespie, J.M. (1963b). Aust. J. Biol. Sci. 16:259.

Gillespie, J.M. (1964). Aust. J. Biol. Sci. 17:282.

Gillespie, J.M. (1965). In "Biology of the Skin and Hair Growth", [Eds., A.G. Lyne and B.F. Short], p.377, [Angus and Robertson, Sydney].

Gillespie, J.M. and Inglis, A.S. (1965). Comparative Biochem. and Physiol. 15:175.

Gillespie, J.M. and Lennox, F.G. (1953). Biochim. Biophys. Acta 12:481.

Gillespie, J.M. and Lennox, F.G. (1955). Aust. J. Biol. Sci. 8:97.

Gillespie, J.M., O'Donnell, I.J. and Thompson, E.O.P. (1962). Aust. J. Biol. Sci. 15:409.

Gillespie, J.M., Reis, P.J. and Schinckel, P.G. (1964). Aust. J. Biol. Sci. 17:548.

Glauert, A.M., Rogers, G.E. and Glauert, R.H. (1956). Nature 178:803.

Golden, R.L., Whitwell, J.C. and Mercer, E.H. (1955). Text. Res. J. 25:334.

Goldsworthy, Y.E. and Lang, W.R. (1953). J. Text. Inst. 44:T230.

Gordon, M. and MacNab, I.A. (1953). Trans. Faraday Soc. 49:31.

Graham, D.R. and Statham, K.W. (1960). Text. Res. J. 30:136.

Green, D.B. and Happey, F. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:283.

Haly, A.R. (1957). Text. Res. J. 27:82.

Haly, A.R. (1963). Text. Res. J. 33:233.

Haly, A.R. and Griffith, J. (1958). Text. Res. J. 28:32.

Haly, A.R. and Inglis, A.S. (1964). Text. Res. J. 34:562.

Happey, F. and Johnson, A.G. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:189.

Harrap, B.S. and Gillespie, J.M. (1963). Aust. J. Biol. Sci. 16:542.

Hill, R.L. (1965). Adv. Protein Chem. 20:37.

Hirabayashi, K. (1938). Bull. Res. Inst. Synth. Fibres, Japan 3:17.

Hock, C.W., Ramsay, R.C. and Harris, M. (1941). J. Res. Nat. Bur. Stand. 27:181.

Holy, H.W. (1966). In "Fourth Amino Acid Colloquium, London, January 1966", p.40, [Technicon Instruments Company Ltd., Chertsey, Eng.]

Homer, A. (1915). J. Biol. Chem. 22:369.

Horio, M. and Kondo, T. (1953). Text. Res. J. 23:373.

Horio, M., Kondo, T., Sekimoto, K. and Funatsu, M. (1965). Proc. Third Int. Text. Res. Conf., Paris, 1965 2:189.

Horio, M., Kondo T., Sekimoto, K. and Teramoto, A. (1960). Z. Naturforsch.15b:343.

Inglis, A.S., Leaver, I.H. and Lennox, F.G. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 2:121.

Johnson, D.J. and Sikorski, J. (1962). Nature 194:31.

Johnson, D.J. and Sikorski, J. (1965a). Nature 205:266.

Johnson, D.J. and Sikorski, J. (1965b). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:147.

Johnson, D.J. and Speakman, P.T. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:173.

Jones, G. (1961). Aust. J. Biol. Sci. 14:485.

Jones, G. (1966a). J. Text. Inst. 57:T368.

Jones, G. (1966b). J. Text. Inst. 57:T539.

Kassenbeck, P. (1965a). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:135.

Kassenbeck, P. (1965b). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:367.

Kassenbeck, P. and Hagege, R. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:245.

Kassenbeck, P., Jacquemart, J. and Monrocq, R. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:209.

Kidd, F. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:221.

Kimmel, J.R., Markowitz, H. and Brown, D.M. (1959). J. Biol. Chem. 234:46.

King, N.L.R. (1967). M.Sc. Thesis, Australian National University.

Lagermalm, G. (1954). Text. Res. J. 24:17.

Lang, J.M. and Lucas, C.C. (1952). Biochem. J. 52:84.

Lang, W.R. (1958). Text. Res. J. 28:90.

Lang, W.R. and Campbell, W.K. (1966). Nature 211:757.

Laxer, G. and Ross, D.A. (1954). Text. Res. J. 24:672.

Laxer, G., Sikorski, J., Whewell, C.S. and Woods, H.J. (1954). Biochim. Biophys. Acta 15:174.

Laxer, G. and Whewell, C.S. (1954). Chem. and Ind. p.127.

Laxer, G. and Whewell, C.S. (1955). Proc. Int. Wool Text. Res. Conf., Aust., 1955 F:186.

Laxer, G., Whewell, C.S. and Woods, H.J. (1954). J. Text. Inst. 45:T482.

Leach, S.J., Rogers, G.E. and Filshie, B.K. (1964). Arch. Biochem. Biophys. 105:270.

Leveau, M. (1956a). Bull. Inst. Text. France 60:61.

Leveau, M. (1956b). Bull. Inst. Text. France 63:91.

Leveau, M. (1957). c.r. Acad. Sci. Paris 244:3183.

Leveau, M. (1958). Bull. Inst. Text. France 74:75.

Leveau, M. (1959a). Bull. Inst. Text. France 79:61.

Leveau, M. (1959b). Bull. Inst. Text. France 79:79.

Leveau, M. (1959c). Bull. Inst. Text. France 80:57.

Leveau, M. (1959d). Bull. Inst. Text. France 80:65.

Leveau, M. (1959e). Bull. Inst. Text. France 85:57.

Leveau, M., Cebe, N. and Parisot, A. (1953). Bull. Inst. Text. France 42:7.

Lindley, H. (1947). Nature 160:190.

Lockart, L.W. (1960). J. Text. Inst. 51:T295.

Louw, D.F. (1960a). Text. Res. J. 30:462.

Louw, D.F. (1960b). Text. Res. J. 30:606.

Lundgren, H.P. (1955). Proc. Int. Wool Text. Res. Conf., Aust., 1955 F:200.

Lundgren, H.P. and Ward, W.H. (1962). Arch. Biochem. Biophys. Suppl. 1:78.

Lundgren, H.P. and Ward, W.H. (1963). In "Ultrastructure of Protein Fibres", [Ed., R. Borasky], p.39, [Academic Press, New York].

Lustig, B., Kondritzer, A.A. and Moore, D.H. (1945). Arch. Biochem. 8:57.

McMurtrie, W. (1886). "Examination of Wools and Other Animal Fibres", [Govt. Printing Office, Washington].

Matoltsy, A.G. (1953). Expt. Cell. Res. 5:98.

Mellor, J.W. (1923). "A Comprehensive Treatise on Inorganic and Theoretical Chemistry", Vol. 3, p.558, [Longmans, London].

Menkart, J. (1966). Private communication.

Menkart, J. and Coe, A.B. (1958). Text. Res. J. 28:218.

Mercer, E.H. (1953). Text. Res. J. 23:388.

Mercer, E.H. (1954a). J. Text. Inst. 45:T365.

Mercer, E.H. (1954b). Text. Res. J. 24:39.

Mercer, E.H. (1954c). Text. Res. J. 24:835.

Mercer, E.H. (1954d). Biochim. Biophys. Acta 15:293.

Mercer, E.H. (1954e). J. Text. Inst. 45:T719.

Mercer, E.H. (1957). Text. Res. J. 27:860.

Mercer, E.H. (1961). "Keratin and Keratinization", p.268, [Permagon Press, Oxford, New York, London and Paris].

Mercer, E.H. , Golden, R.L. and Jeffries, E.B. (1954). Text. Res. J. 24:615.

Mercer, E.H., Munger, B.L., Rogers, G.E. and Roth, S.I. (1963). Nature 201:367.

Miro, P. (1961). Bull. Inst. Text. France 94:65.

Miro, P. and Blade, J. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:507.

Nott, J.A. and Sikorski, J. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:197.

Ohara, K. (1938). Melliand Textilber. 19:407.

Ohara, K. (1939). Melliand Textilber. 20:326.

Olcott, H.S. and Fraenkel-Conrat, H. (1947). J. Biol. Chem. 171:583.

O'Shea, J.M. (1967). Private communication.

Oster, G. and Yamamoto, M. (1963). Chem. Rev. 63:257.

Parisot, A., Allard, N. and Baures, M. (1965). Proc. Third Int. Wool Text. Res. Conf., Paris, 1965 1:421.

Perutz, M.F. (1965). J. Mol. Biol. 13:646.

Piez, K.A. and Morris, L. (1960). Anal. Biochem. 1:187.

Priestley, G.C. (1966). J. Text. Inst. 57:T438.

Race, E. (1946). In "Symposium on Fibrous Proteins", p.67, [Soc. Dyers and Colourists, Bradford, Eng.]

Ritter, R. and Reumuth, H. (1960). Z. ges. Textil-Industrie 62:484 and 578.

Ritter, R., Reumuth, H. and Tomopulos, K. (1960). Z. ges. Textil-Industrie 62:342.

Ritter, R, and Tomopulos, K. (1959). Naturwissenschaften 46:234.

Rogers, G.E. (1958). Biochim. Biophys. Acta 29:33.

Rogers, G.E. (1959a). J. Ultrastructure Res. 2:309.

Rogers, G.E. (1959b). Ann. N.Y. Acad. Sci. 83:378.

Rogers, G.E. (1962). Nature 194:1149.

Rogers, G.E. (1964). In "The Epidermis", [Eds., W. Montagna and W. C. Lobitz Jr.], p.179, [Academic Press, New York and London].

Rogers, G.E. and Filshie, B.K. (1962). Fifth Int. Congress for Electron Microscopy, Philadelphia, 1962 O:2.

Rogers, G.E. and Filshie, B.K. (1963). Quoted in Leach, Rogers and Filshie (1964).

Ross, D.A. and Speakman, J.B. (1957). Text. Res. J. 27:345.

Royer, G.L. and Millson, H.E. (1940). Amer. Dyestuff Reptr. 29:697.

Rudall, K.M. (1936). Ph.D. Thesis, University of Leeds. Quoted in Rudall (1955a).

Rudall, K.M. (1955a). Proc. Int. Wool Text. Res. Conf., Aust., 1955 F:9.

Rudall, K.M. (1955b). Proc. Int. Wool Text. Res. Conf., Aust., 1955 F:176.

Ryder, M.L. (1956). Nature 178:1409.

Ryder, M.L. (1958). Proc. Roy. Soc. Edin. 67B:65.

Ryder, M.L. (1959). Quart. J. Microsc. Sci.100:1.

Ryder, M.L. (1963). In "Fibre Structure", [Eds., J.W.S. Hearle and R. H. Peters], p.534, [The Textile Institute and Butterworths, Manchester and London].

Satlow, G. (1959). Textil-Rundschau 14:446.

Satlow, G. and Kessler, H. (1958). Text. Res. J. 28:359.

Schoberl, A. (1942). Klepzigs Textil-Z. 45:41.

Schoberl, A. (1960). Melliand Textilber. 41:188.

Schroeder, W.A., Kay, L.M., Lewis, B. and Munger, N. (1955). J. Amer. Chem. Soc. 77:3901.

Shah, S.M.A. and Whiteley, K.J. (1966). Text. Res. J. 36:586.

Simmonds, D.H. (1954). Aust. J. Biol. Sci. 7:98.

Simmonds, D.H. (1955). Proc. Int. Wool Text. Res. Conf., Aust., 1955 C:65.

Simmonds, D.H. (1958). Text. Res. J. 28:314.

Simmonds, D.H. and Bartulovich, J.J. (1958). Text. Res. J. 28:378.

Slinger, R.I., Smuts, S. and Mellet, P. (1966). Text. Res. J. 36:1023.

Snaith, J.W. (1960). Text. Res. J. 30:543.

Snyman, J.G. (1963a). Text. Res. J. 33:217.

Snyman, J.G. (1963b). Text. Res. J. 33:803.

Spearman, R.I. and Barnicot, N.A. (1960). Amer. J. Phys. Anthrop. 18:91.

Stein, W.D. (1955). Nature 175:256.

Suarez, K. (1966). Melliand Textilber. 47:671.

Swift, J.A. and Holmes, A.W. (1965). Text. Res. J. 35:1014.

Thompson, E.O.P. and O'Donnell, I.J. (1959). Aust. J. Biol. Sci. 12:282.

Thompson, E.O.P. and O'Donnell, I.J. (1964). Aust. J. Biol. Sci. 17:277.

Thorsen, W.J. (1958). Text. Res. J. 28:185.

Vogel, A.I. (1956). "A Textbook of Practical Organic Chemistry", [Third edit.], p.453, [Longmans, London].

Ward, W.H. and Bartulovich, J.J. (1955). Text. Res. J. 25:888.

Ward, W.H. and Bartulovich, J.J. (1956). J. Phys. Chem. 60:1208.

Ward, W.H. and Bartulovich, J.J. (1964). Tech. Wool Conf. [ARS-74-29], U.S. Dept. of Agriculture, p.85.

Ward, W.H., Binkley, C.H. and Snell, N.S. (1955). Text. Res. J. 25:314.

Watkins, W.H., Royer, G.L. and Millson, H.E. (1944). Amer. Dyestuff Reptr. 33:52.

Watt, I.C. and Morris, R. (1967). J. Text. Inst. 58:84.

Williams, V.A. (1962). Text. Res. J. 32:83.

Woods, H.J. (1935).J. Text. Inst. 26:T93.

Woods, H.J. (1938). Proc. Roy. Soc. A166:76.

Zahn, H. and Haselmann, H. (1950). Melliand Textilber. 31:225.

Zuber, H., Traumann, K. and Zahn, H. (1955). Proc. Int. Wool Text. Res. Conf., Aust., 1955 C:127.